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493. CONCERNING THE INTERPRETATION OF OBSERVATIONS, OBTAINED FROM RECORDS AND EXPERIMENTS, ON MILKING DAIRY COWS

BY C. P. COX

National Institute for Research in Dairying, University of Reading

(With 1 Figure)

INTRODUCTION

A number of important general considerations affect the ultimate value of records and results obtained from observations on the milk production of dairy cattle; much depends, for example, on whether experiments are primarily directed towards the elucidation of fundamental physiological problems and/or are part of programmes of applied research leading to recommendations in husbandry. No exhaustive discussion of such general considerations is to be attempted here, although some may later be introduced in connexion with the problem of interrelated responses or observations.

Aspects of this question, which basically concerns the relation between the yield of milk and its composition, have been previously discussed by a number of authors. For example, a formula for fat-corrected milk was proposed by Gaines & Davidson (1), questions of inheritance have been discussed by Buchanan Smith (2) and Mahadevan (3), and Edwards (4) has recently stated that there is a fundamental inverse relationship between the yields of milk and butterfat. The formula for fat-corrected milk and the approach represented by Edwards (4) illustrate the different methods currently used in the treatment of records and experimental results.

A STATEMENT OF THE PROBLEM

The principal quantities commonly concerned in records or experimental results are given below together with the notation to be used:

y = yield of the aqueous constituent in lb.,
x = yield of fat in lb.,
z = yield of solids-not-fat in lb.,
Y = total 'milk' yield in lb.,
p = percentage of butterfat,
q = percentage of solids-not-fat.

The quantity y is strictly defined by equation (1) below as the remainder after the yields of fat and of solids-not-fat have been subtracted from the total yield. For subsequent simplicity this aqueous constituent will be termed 'water'. In cases where the yield of solids-not-fat is unknown the term 'serum' will be used to denote fat-free milk, that is, together, the solids-not-fat and aqueous constituents.

The total milk yield, Y, is usually given by weight, measurements being obtained to about the nearest $\frac{1}{4}$ lb., although the accuracy varies according to circumstances. Volume measurements are also used in practice, but for present purposes weights alone will be considered. Direct determinations of fat yield and of the yield of solids-not-fat are

generally impracticable, and instead estimates of these yields are obtained as products of the milk yield with estimates of the corresponding percentages in samples.

Questions associated with possible inaccuracies in sampling procedures, measurements, etc., will be ignored here, and it will be assumed, sufficiently for present purposes, that the observations are accurate. Accordingly, the situation is that, of the above quantities, x, y and z may be regarded as the fundamental ones, while Y, p and q are those directly observable. The simple relations (1), (2) and (3) below connect the sets of quantities, and the problem to be considered is that of obtaining full and clear information from observed results:

$$Y = x + y + z, \tag{1}$$

$$p/100 = x/(x+y+z) = x/Y, \tag{2}$$

$$q/100 = z/(x+y+z) = z/Y. \tag{3}$$

Corresponding relations obtain between other quantities such as total solids and solids-not-fat percentage of fat-free milk, but these quantities are not so commonly used as those above.

For convenience in subsequent treatment, Y, p and q will be termed 'derived quantities'.

EXPOSITION

It is seen that, using the above equations, all of the six quantities Y, p, q, x, y and z may be calculated from any three of them and in particular, therefore, that the derived quantities give no further information than that contained in results for x, y and z. The derived quantities are, however, the ones most frequently used in present practice, one of the reasons for this being that they form the directly observable experimental or record data. The difficulties in interpretation arise from the fact that changes in a derived quantity can occur as a result of independent or associated changes in its constituents. In particular, for example, the butterfat percentage may be affected following changes in either or both of its numerator and denominator. Further, it is clear that compensating changes in constituents may occur such that no change is apparent in a derived quantity, and accordingly results discussed solely in terms of the derived quantity may not give all the available information.

Most of these difficulties can be avoided if results are discussed in terms of the quantities x, y and z, and it will be shown that such discussion is generally advantageous in both fundamental and practical studies. Further, if in particular applications it is desired to examine changes in the percentage composition, it is possible to show relations between these changes and the associated changes in the fundamental quantities. The formulae for this analysis, which may be conveniently termed a constituent or dilution analysis, are as follows, wherein suffixes 1 and 2 denote the initial and final values of the quantities respectively:

$$Y_2 - Y_1 = (x_2 - x_1) + (y_2 - y_1) + (z_2 - z_1), \tag{4}$$

$$\frac{(p_2-p_1)}{100} = \frac{(y_1+z_1)(x_2-x_1)}{(x_1+y_1+z_1)(x_2+y_1+z_1)} - \frac{x_1(y_2-y_1)}{(x_1+y_1+z_1)(x_1+y_2+z_1)} - \frac{x_1(z_2-z_1)}{(x_1+y_1+z_1)(x_1+y_1+z_2)}$$
$$-(y_2-y_1)\left[\frac{(x_2-x_1)}{(x_1+y_1+z_1)(x_1+y_2+z_1)} - \frac{x_1(2x_1+y_1+y_2+2z_1)(x_2-x_1+z_2-z_1)}{(x_1+y_1+z_1)^2(x_1+y_2+z_1)^2}\right] \tag{5}$$
$$+\text{product terms, in practice usually of higher order.}$$

The similar formula for $q_2 - q_1$ is obtained by interchanging x and z in (5).

The formulae are simply the mathematical expressions of the effects of independent changes, and in themselves of course they give no information about the process of secretion. The first term on the right-hand side of (5), for example, represents the change in the butterfat ratio which would occur if, without changing the water or the solids-not-fat contents, the butterfat yield were increased from x_1 lb. to x_2 lb. The second and third terms express similar changes consequent on single changes in the yields of water and solids-not-fat respectively. When such changes are simultaneous the total effect on the butterfat percentage will clearly differ from the sum of the separate effects, and the difference may formally be described as the residual or interaction term. In practice the largest residual contributions are generally derived from the product terms involving the change in the yield of water with the first powers of the other changes, and this amount may be calculated from the fourth term in the above formula. In application, however, the calculations can conveniently be arranged vertically and the total residual term obtained by subtraction. As a check it may be noted that the sign of the residual term will generally be positive when the changes in the water and butterfat yields are of opposite signs. In practice many of the changes are small, and in this case equation (5) simplifies with slight approximation to

$$\frac{(p_2-p_1)}{100}=\frac{(y_1+z_1)\,(x_2-x_1)}{(x_1+y_1+z_1)^2}-\frac{x_1(y_2-y_1)}{(x_1+y_1+z_1)^2}-\frac{x_1(z_2-z_1)}{(x_1+y_1+z_1)^2}+\text{ residual,} \tag{6}$$

wherein the first terms all have the same denominator.

On the assumptions of the above formulae it is clear that the respective contributions of changes in the yields of butterfat, water and solids-not-fat, to the changes in butterfat percentage will not in general be equal. From (6) the relative power of changes in the yields of butterfat and water to affect the butterfat percentage may be expressed approximately as

$$-\frac{(y_1+z_1)}{x_1}\times\frac{(x_2-x_1)}{(y_2-y_1)}=-\left(\frac{100}{p}-1\right)\times\frac{(x_2-x_1)}{(y_2-y_1)}. \tag{7}$$

This shows that a butterfat percentage of 3·6, for example, would be depressed if an increase in the yield of water exceeded about 27 times a simultaneous increase in the yield of butterfat. It is, of course, easy to see that if opposing changes in the yields of milk and of butterfat are proportional to those yields respectively, then the butterfat percentage will be unaltered, so that conclusions based solely on considerations of the butterfat percentage may be at least incomplete and possibly misleading. Similar formulae and arguments apply to the percentage of solids-not-fat—a solids-not-fat percentage of about 9, for example, would be depressed by an increase in water yield exceeding about 10 times a simultaneous increase in the yield of solids-not-fat.

And although the above considerations and the applications of the formulae will depend on the magnitudes of the various yields and changes in particular cases, as will be seen in the following examples, it appears generally more useful to discuss changes in the constituent yields rather than in the percentages alone. Changes in the percentages may then be examined using the constituent analysis as defined above.

EXAMPLES

1. In an experiment reported by Balch, Balch, Bartlett, Cox & Rowlands (5), the effects on milk composition of varying the amounts of hay in the diet were investigated. The following observations in the treatment period were obtained after adjustment for initial differences:

		Amount of hay (lb./day)		
		18 (control)	6	2
Milk yield (lb./day)	Y	28·65	31·06	27·11
Fat percentage	p	3·77	3·28	3·18
Solids-not-fat percentage	q	8·97	8·92	8·95
Hence we have:	y	25·00	27·27	23·82
	x	1·08	1·02	0·86
	z	2·57	2·77	2·43

Consider now the effects of the 6 lb./day treatment compared with the 18 lb./day control treatment. For the changes in x, y and z we have in pound units

$$x_2-x_1=-0{\cdot}06, \quad y_2-y_1=2{\cdot}27, \quad z_2-z_1=0{\cdot}20, \quad Y_1=x_1+y_1+z_1=28{\cdot}65.$$

The contributions of the changes in butterfat yield, water yield and yield of solids-not-fat, to the total change in the butterfat percentage, $-0{\cdot}49\,\%$, may now be obtained from (5) as:

Constituent		Change in butterfat percentage
Butterfat	$-(100\times27{\cdot}57\times0{\cdot}06)/(28{\cdot}65\times28{\cdot}59)$	−0·195
Water	$-(100\times1{\cdot}08\times2{\cdot}27)/(28{\cdot}65\times30{\cdot}92)$	−0·277
Solids-not-fat	$-(100\times1{\cdot}08\times0{\cdot}20)/(28{\cdot}65\times28{\cdot}85)$	−0·261
Residual		+0·008
	Total	−0·490

Similarly for the comparison of the 2 lb./day treatment with the control treatment there is a decrease of 0·59 in the butterfat percentage for which the constituent analysis is

Constituent	Change in butterfat percentage
Butterfat	−0·745
Water	+0·162
Solids-not-fat	+0·019
Residual	−0·026
Total	−0·590

The application of the corresponding analysis to the changes in the percentage of solids-not-fat gives the following table:

Constituent	18 lb./day–6 lb./day	18 lb./day–2 lb./day
Butterfat	+0·019	+0·069
Water	−0·659	+0·385
Solids-not-fat	+0·631	−0·447
Residual	−0·041	−0·027
Total	−0·050	−0·020

Considering first the butterfat percentage, the preceding analysis on the dilution model shows that, for the 6 lb./day hay ration the increase in the yield of water alone would contribute $0{\cdot}28\,\%$, i.e. slightly more than the contribution, $0{\cdot}20\,\%$, from the decrease in butterfat yield, to the depression of the butterfat percentage from the figure for the control treatment. For the 2 lb./day ration the change in the yield of butterfat would contribute $0{\cdot}74\,\%$ to the depression as against a much smaller and opposing contribution from the change in the yield of water. In both cases the contributions from changes in the yield of solids-not-fat would be relatively small. A further point which this analysis

reveals is an apparent qualitative difference between the effects of the non-control treatments, in that were it possible to place the results on a common basis such as that of energy, whereas the effect of the 6 lb./day treatment could perhaps be regarded as a partial re-direction of energy from the butterfat fraction into other components, on the 2 lb./day treatment the yields of all components are reduced, suggesting, if other things can be assumed equal, a reduction of net output however estimated, and one possibly associated, for example, with underfeeding.

The dilution analysis for the solids-not-fat percentage again shows the importance of the accompanying changes in the yield of water; on the 6 lb./day treatment, for example, an appreciable rise in the yield of solids-not-fat was not reflected in the solids-not-fat percentage. Generally, it is seen that changes in the solids-not-fat yield have little effect on the butterfat percentage, and conversely; the residual terms are accordingly largely attributable to product terms of water changes with changes in the factor being considered.

All the information as elucidated above is of course contained in the table of values for x, y and z; the utility of the present analysis lies in obtaining quantitative values for the constituent contributions.

Table 1. *Lactation changes in yield and composition*

Stage of lactation (days)	Milk yield p.m. (lb.)	Fat (%)	Yield of fat-free serum (lb.)	Yield of fat (lb.)
0–30	14·67	5·87	13·809	0·861
31–	14·58	5·87	13·724	0·856
61	13·59	5·83	12·798	0·792
91	12·24	5·91	11·517	0·723
121	11·07	5·93	10·414	0·656
151	10·35	5·96	9·733	0·617
181	9·45	6·09	8·874	0·576
211	8·55	6·20	8·020	0·530
241	7·74	6·31	7·252	0·488
271	7·02	6·44	6·568	0·452
301	6·57	6·51	6·142	0·428

2. Some further points may be illustrated by the consideration of parts of the paper by Edwards (4) dealing with the relation between milk yield and butterfat percentage and the influences of a number of factors on these quantities. It is stated that there is a fundamental inverse relationship between the milk yield and butterfat percentage, and from analyses of records from two dairy herds, linear regressions within season and stage of lactation, of butterfat percentage on milk yield have been obtained. These regression equations have then been used to obtain values of butterfat percentage corrected for milk yield, and the effects of, for example, season upon these corrected values have been discussed.

The validity and usefulness of this process may, however, be limited by the dual nature of the relationship between butterfat percentage and total milk yield. For besides the direct mathematical connexion (2) there is the possible association between the yields of milk and butterfat consequent on the physiological economy of the animal itself, and the latter may not be consistent within seasons and lactations. Both factors can, however, rightly be described in some sense as fundamental, and both will affect the calculated regressions. An approximate mathematical treatment has also indicated that a quadratic term may be desirable in a regression formulation of (2) alone. On p. 104 Edwards discusses the nature of the relationship between milk and fat percentage

and concludes that 'the factors controlling (*a*) the quantity of milk (i.e. the rate of secretion per unit of time) and (*b*) the fat percentage, are separate' and inversely related. This conclusion appears to have been reached from consideration of a table giving the rates of secretion for evening and morning milk yields and corresponding fat percentages at successive stages of lactation. The evening figures are shown in Table 1, and the yields of fat-free serum and butterfat calculated from them are given in the last two columns. The figures refer to records of cows tested for entry into the English Jersey Cattle Club Register of Merit, 1925–32. The last two columns show that the same figures are equally consistent with other hypotheses, e.g. that some factors influence both the yield of fat and of fat-free serum so that both decline as lactation advances.

The first part of the conclusion above—that the factors controlling the quantity and fat percentage are separate, though perhaps true, cannot be deduced from the data alone. The remainder of the conclusion and the particular alternative hypothesis both in fact derive from tautological descriptions of the observed phenomena. Simply on the basis of agreement with the phenomena, therefore, more fundamental or physiological validity cannot logically be claimed for either hypothesis.

The yields of butterfat and the corresponding yields of butterfat-free serum, ignoring stage of lactation, are plotted on the accompanying graph (Fig. 1) for both morning and

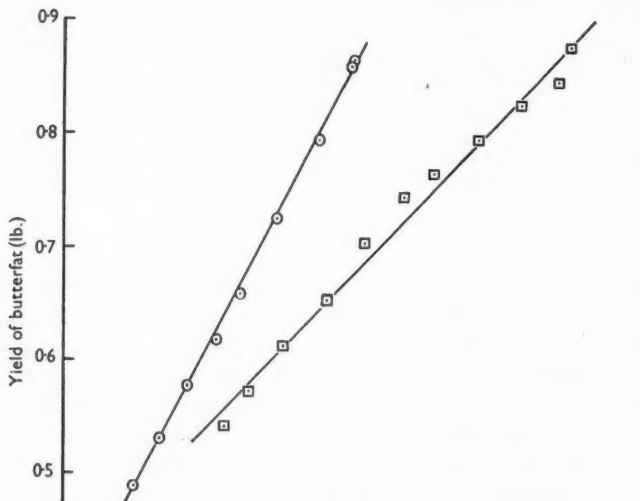

Fig. 1. Butterfat and serum yields, English Jersey Cattle Club records, 1925–32.

evening milks. Although the curvatures are in fact significant, it is apparent that to a first approximation the relations are almost linear. This is somewhat remarkable, and it is hoped to discuss it further in an investigation, using N.I.R.D. records, of relations between the yields of milk constituents.

From the point of view under development here a number of questions discussed in the paper by Edwards (4), and particularly those associated with a simple difference effect, may be treated adequately and informatively in terms of the yields rather than ratios of constituents. Consider, for example, the difference between the fat concentrations of morning and evening milks. On p. 104 the mean values for morning and evening milk yields and fat percentages are used, together with the regression equations, to establish the statement that the difference in these fat concentrations of morning and evening milks is not explained by the increased yield of milk in the morning. The means are as follows:

	Evening	Morning	Difference
Milk yield (lb.)	10·7	16·1	+5·4
Fat percentage	6·12	4·53	−1·59

and the corresponding fundamental quantities are, therefore,

x (lb.)	0·655	0·729	+0·074
$y+z$ (lb.)	10·045	15·371	+5·326

In the absence of figures for solids-not-fat the constituent analysis is formally obtained from equation (5) by putting $z_2=z_1=0$, and letting y now represent the yield of butterfat-free serum. If, in fact, the two yields of solids-not-fat are equal, the serum difference will be equivalently a difference in the yields of the aqueous constituents. The analysis then becomes:

Constituent		Change in butterfat percentage
Butterfat	$(100\times 10{\cdot}045\times 0{\cdot}074)/(10{\cdot}7\times 10{\cdot}774)$	+0·645
Serum	$-(100\times 0{\cdot}655\times 5{\cdot}326)/(10{\cdot}7\times 16{\cdot}026)$	−2·034
Residual		−0·201
	Total	−1·590

Edwards' statement above may be compared with the interpretation of this analysis, which shows that most of the decrease in the fat percentage from evening to morning milk directly corresponds to the increased yield of serum. Calculation of the first term of the expression for the residual term in (5) gives

$$-(100\times 5{\cdot}326\times 0{\cdot}074)/(10{\cdot}7\times 16{\cdot}026)=-0{\cdot}230\,\%,$$

which is very close to the residual term $-0{\cdot}201\,\%$ obtained by subtraction in the above analysis. Altogether then it appears that the addition of 5·326 lb. of serum and 0·074 lb. fat to 10·7 lb. of milk of 6·12% fat would result in 16·1 lb. of milk of 4·53% fat. The addition of 5·326 lb. of serum alone would more than account for the diminished butterfat percentage. It would reduce the fat percentage by 2·034%, i.e. to 4·09% approximately. It should be noted that additional evidence on the 'night effect' is presented in Edwards (4) and Campbell (9). The preceding argument itself is given not to demonstrate the absence of the effect but to show that, contrary to indications from the regression method, the particular figures above cannot demonstrate the 'night effect'. If the butterfat yields in two cases are equal, it is clear from elementary considerations that differences in the corresponding butterfat percentages are explicable in terms of differences in serum yield. If, as in the above example, the morning butterfat yield exceeds the evening butterfat yield, a lower morning butterfat percentage is necessarily associated with a higher serum yield.

3. Another application of the constituent analysis may usefully arise in considering comparisons between productions such as those of different breeds, where different levels of the milk constituents are encountered. For illustration, figures from the 1950 Report

of the Production Division M.M.B. (6), referring to the average yields of milk and butterfat of herds in the recording scheme, are used, and it will be sufficient here to consider only the three breeds with the largest numbers of recorded lactations. The figures required are:

Breed or breed type	Average yield lb.		Average butterfat percentage
	Milk	Butterfat	
Ayrshire	8088	308	3·81
Friesian	9141	315	3·45
Shorthorn	7365	264	3·58

Constituent analyses for the comparison between pairs of breeds may now be obtained as in the previous paragraph. For example, comparisons of the Ayrshire with the Friesian and with the Shorthorn figures give:

Friesian—Ayrshire		Shorthorn—Ayrshire	
Constituent	Change in butterfat percentage	Constituent	Change in butterfat percentage
Butterfat	+0·083	Butterfat	−0·526
Fat-free serum	−0·436	Fat-free serum	+0·349
Residual	−0·007	Residual	−0·053
Total	−0·360	Total	−0·230

The generality of remarks on the interpretation of the above analyses is necessarily conditioned by the fact that the mean figures above refer to that herd selection implicit in the recording scheme. Further, the comparison obtained here is one of output only, whereas a more complete economic assessment will clearly depend on other factors such as the relative efficiencies of food conversion, and of utilization of management (themselves influenced by factors such as animal size) and on the weights, real and apparent, given to various milk constituents. In connexion with this weighting there are differences between the nutritional values of the fats and of the solids-not-fats; the water yield may also be important, apart from its effect on transport costs, in that whilst it is a legal offence for a human to add any water to milk intended for human consumption, and whilst milk is bought from farmers on a simple yield basis, increased water yield permits wider distribution of milk nutrients and greater financial returns to producers.

The analyses of the particular figures above, however, show that the difference between the Ayrshire and Friesian butterfat percentages is almost entirely accountable in terms of the difference in the yield of fat-free serum, and that if positive weights are assigned to the constituents, the Friesian net output exceeded the Ayrshire figure. A different situation obtains in the comparison of the Shorthorn and Ayrshire butterfat figures. There is a depression of about 0·5% corresponding to an appreciably diminished butterfat yield, but this is largely compensated by a decrease in the yield of milk serum. It may be noted that the former exceeds the depression, 0·4%, attributed by the formula to the increase in the Friesian milk serum, and although the mean Shorthorn net output was the lowest of the three considered, the butterfat percentage exceeded that of the Friesian.

4. Considerations similar to those above will apply also in discussions concerning changes in milk quality with time (season, stage of lactation, etc.), on which, in connexion with solids-not-fat, numerous papers have been published, for example, McLean (7). As before, and as stated by, for example, Edwards (4) (Introduction), analyses solely in terms of the percentages of butterfat, solids-not-fat, etc., may be insufficient unless accompanied by parallel treatment of corresponding changes in milk yield.

DISCUSSION

The detailed discussions of the examples above illustrate some of the alternative interpretations which can reasonably be placed on experimental and record data, and it is clear that a single standardized response valid for making comparisons between the yields of animals under different circumstances would be of great value. An early method of such standardization introduced by Gaines & Davidson (1) is commonly used, and some further points may be illustrated by considering it. Apart from the method, however, the above paper includes some valuable discussions, in similar general terms, of many of the questions dealt with in the present study.

Basically the method itself consists of converting the yield of milk into an energy equivalent and dividing this value by the energy of 1 lb. of milk of 4% fat content to obtain the yield of fat-corrected milk. The fundamental hypothesis adopted is that the total energy value of milk is constant if all factors other than composition are equalized. The truth or otherwise of this hypothesis is not here questioned, but an ensuing statement, the sentiments of which have appeared in far more recent literature—'what we really have to consider is the influence of composition on milk yield'—merits deeper examination. A particular cause-effect relationship is implied for which even now the physiological evidence is not unequivocal. Should the statement become substantiated, formulae corresponding to those previously given can readily be obtained to distinguish the separate effects on fat yield of fat percentage and milk yield. It is not beyond possibility, however, that the relationship is more complex and neither in complete agreement nor disagreement with that implied by the statement, in which case the alternative approach as developed in the present paper has advantages. In terms of energy it can be regarded as based on a distribution of available energy between the various milk constituents—the fundamental qualities, controlled by factors which need not be specified at present but which do not necessarily impose physiological cause-effect relationships between any of the constituents. With this approach, for example, some of the correlation observed between the percentages of butterfat and of solids-not-fat may be attributed to the fact that the respective yields are expressed in percentages of the same denominator.

Apart from considerations such as these there remains no outstanding question of principle affecting the use of the Gaines & Davidson formula. In the light of more recent knowledge, however, some differences in the final formula might be expected, to include, for example, an explicit term for solids-not-fat.

The situation developed so far is that some currently used methods of assessing the production of dairy cattle have been examined and compared in practical examples with a proposed alternative method. Using this alternative, based on the yields rather than on the percentages of the milk constituents, and an ancillary constituent analysis, it is possible to make more detailed interpretations of experimental results and record data than those commonly obtained.

It is apparent that the problem has a number of different aspects in that relations between the initial input and the final responses may be considered as part of different though not necessarily independent economies. These include, for example, the efficient utilization of labour and feeding stuffs in the production of nutritive material, and within this there are the economies of the animal itself as fundamental physiological processes.

Yields of fat and of solids-not-fat may be expected to be the important factors in animal-feeding policies, but the heritability of the butterfat percentage makes it the more important factor in animal breeding. At present also some weight is attributable to the water yield in that it permits wider distribution of nutrients in liquid milk. Further, in fundamental research on physiological processes, where it is frequently desirable to obtain maximum discrimination between one output and another independent of utility, some combination of all the constituents may be the most effective.

Apart from the fact that not all considerations exemplified above are capable of quantitative expression, it may be seen that no single arithmetical expression of the response can be expected to meet all requirements and circumstances. Accordingly, a general thesis emerging from the present study is that the responses might ideally be expressed in terms of some functions of the fundamental and/or derived variables, including all the information they contain, the form of which functions may vary with the requirements of particular circumstances. Such a function, sometimes called a transformation function, may be written in the case of dairy cattle responses as

$$R_i = \phi_i(x, y, z, p, q, w, \text{etc.}),$$

where R_i is the response relevant to, say, the ith set of circumstances. Changes in the quantities such as x, y and z may or may not, according to the magnitudes of the changes and their expression in the formula, give rise to changes in R, and R is used as the basis for the appropriate interpretations and decisions.

For illustrative purposes only, consider the expression

$$R = \lambda_1 + \lambda_2 x + \lambda_3 y + \lambda_4 z + \frac{\lambda_5 x + \lambda_6 y}{x + y + z} + \lambda_7 w, \tag{12}$$

w being the live weight. Here the λ's are coefficients which when constant may be regarded as defining a family of circumstances in each of which R is a constant interpreted as, for example, an output, energy, or value.

The Gaines & Davidson formula previously referred to may be obtained from (12). For, as originally written,

$$\text{F.C.M.} = 0{\cdot}4\,M + 15\,F, \tag{13}$$

where F.C.M. = fat corrected milk in lb., M = observed milk yield in lb., F = fat content in lb., becomes in the present notation

$$\begin{aligned} \text{F.C.M.} &= 0{\cdot}4(x + y + z) + 15x \\ &= 15{\cdot}4x + 0{\cdot}4y + 0{\cdot}4z. \end{aligned} \tag{14}$$

This corresponds to (12) with

$$\lambda_1 = 0, \quad \lambda_2 = 15{\cdot}4, \quad \lambda_3 = \lambda_4 = 0{\cdot}4, \quad \lambda_5 = \lambda_6 = \lambda_7 = 0,$$

R now being interpreted as the yield of fat-corrected milk, claimed by the authors to be relevant to cases where a physiological comparison is required, and discussed also in connexion with feeding costs.

Another example is obtained from the points scheme used in judging milk production at the London annual dairy show. Ignoring for present purposes deductions, and points for stage of lactation, points have been awarded as follows:

one point for every pound of milk given during 24 hr.,
twenty points for every pound of fat in the milk,
four points for every pound of solids-not-fat in the milk.

i.e. $$\text{Milking points} = x + y + z + 20x + 4z$$
$$= 21x + y + 5z, \quad (15)$$

corresponding to (12) with $\lambda_2 = 21$, $\lambda_3 = 1$, $\lambda_4 = 5$ and all other λ's $= 0$. It is interesting to compare the ratios of the coefficients of fat and water yield in the two cases. In (14) we have a ratio of $15{\cdot}4/0{\cdot}4 = 38{\cdot}5$, as against 21 in (15), so that it is possible for an order established on the somewhat arbitrary basis of milking points to be reversed if fat-corrected yields calculated from the Gaines & Davidson formula are used. (In the 1947 milking trials (8), animal 126, a British Friesian, scored 200·00 milking points as against 190·54 scored by animal 247, an Ayrshire; their fat-corrected milk yields were 92·02 and 96·13 lb. respectively).

The basic general problems are now seen to be the determination of appropriate transformation formulae and the definition of the corresponding circumstances. It would be useful also to define the circumstances in which simple practical expressions such as total solids per cent ($\lambda_5 = \lambda_6 = 100$, all other λ's $= 0$) are most relevant. In many cases, however, it may not be possible to obtain satisfactory formulae in simple terms, particularly when differing subjective assessments of the relative importance of different response factors enter. Accordingly, in many circumstances in which the responses of dairy cattle are interpreted, the particular immediate recommendations are that where possible the yields of water (or total milk), butterfat and solids-not-fat should be examined and quoted separately (in all feeding experiments body-weight changes, clearly a part of the output, should also be included); and where, for practical purposes, interest lies in percentages of composition fractions, the above analysis may conveniently be supported by a constituent or dilution analysis of the type given in the second paragraph.

SUMMARY

1. Some questions concerning the interpretation of observations on milking dairy cattle are considered.

2. A formula is presented for analysing the contributions of changes in the numerator and denominator to the changes in a percentage or fraction, and the formula is applied to changes in the composition of milk.

3. The depressions of the butterfat and solids-not-fat percentages consequent on diets reduced in hay in a dairy-cattle feeding experiment are examined with this constituent analysis.

The contributions of changes in the magnitudes of the aqueous, butterfat and solids-not-fat constituents to the difference between the butterfat percentages of cows on two treatments, 18 lb. hay/day (control) and 6 lb. hay/day, are about equal. There is an apparent qualitative difference between the effects of the two non-control treatments, and a reduced yield of butterfat would alone largely account for the lower butterfat percentage of cows on the 2 lb. hay/day treatment.

The small reductions in the percentages of solids-not-fat are accountable by almost equal but opposing effects of differences in the yields of the aqueous and solids-not-fat constituents.

4. A simple relationship has been found between figures for the yields of butterfat and of butterfat-free serum obtained from records, given in Edwards (4), of animals tested for entry into the English Jersey Cattle Club Register of Merit, 1925–32.

5. Analysis of figures in Edwards (4) discloses that the excess of morning over evening yield of serum is more than sufficient to account for the difference between the morning and evening butterfat percentages quoted. It is not, however, claimed that this finding is generally true.

6. Applied to figures published by the Milk Marketing Board for the milk and butterfat productions of three recorded breed types, the constituent analysis shows that whilst the difference between the Ayrshire and Friesian butterfat percentages is almost entirely accountable in terms of the difference in the yields of butterfat-free serum, the depression of the Shorthorn butterfat percentage from the Ayrshire figure is explicable, on the other hand, as a reduction consequent on a diminished butterfat yield and a partially compensating increase corresponding to a diminished yield of serum.

7. The assessment of the production of dairy cattle is generally discussed, and particular methods considered are the formula for fat-corrected milk and the points scheme used at the London annual dairy show. It is suggested that the yields of water or total milk, of butterfat and of solids-not-fat might with advantage be examined and quoted separately, together, if possible, with body weights. The constituent analysis may then be used to examine changes in the percentage composition of milk.

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REFERENCES

(1) Gaines, W. L. & Davidson, F. A. (1923). *Bull. Univ. Ill. Agric. Exp. Sta.* no. 245.
(2) Buchanan Smith, A. D. (1936). *J. Brit. Dairy Fmrs' Ass.* **48**, 11.
(3) Mahadevan, P. (1951). *J. agric. Sci.* **41**, 94.
(4) Edwards, J. (1950). *J. agric. Sci.* **40**, 100.
(5) Balch, C. C., Balch, D. A., Bartlett, S., Cox, C. P. & Rowland, S. J. (1952). *J. Dairy Res.* **19**, 39.
(6) Anon. (1950). Production Division, M.M.B. Report, no. 1.
(7) McLean, J. W. (1951). *Tech. Publ. Lincoln College, Univ. N.Z.* no. 7.
(8) Drakeley, T. J. (1948). *J. Brit. Dairy Fmrs' Ass.* **52**, 46.
(9) Campbell, J. W. D. (1931). *J. Dairy Res.* **3**, 52.

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494. THE MILK-EJECTION RESPONSE OF THE SOW TO STANDARD DOSES OF OXYTOCIC HORMONE

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The milk-ejecting activity of the hormones of the posterior-pituitary gland is well known and has been adequately reviewed (1). Most of the published information deals with the lactating dairy cow. In the course of work at this Station involving the lactating sow as a basis for assay of the milk-ejecting hormones (2,3), data have been collected on the effect of a standard dose of oxytocin. Our normal assay procedure (2) involves giving the animal a preliminary dose of 0·5 unit of oxytocin. Thus over one lactation we have been able to record the time between injection into the ear vein and the onset of milk let-down, and the period during which milk may be obtained by simple hand-milking, for a number of sows. Braude & Mitchell (4) have published figures for the milk let-down effects of doses of oxytocin ranging from 1 to 10 units. The following results were obtained with 0·5 unit. All sows used were of the Berkshire breed.

EXPERIMENTAL

The procedure adopted for all milk let-down assays was as follows. The sow was brought into a milking stall and the mammary glands washed. Oxytocin solution (0·5 unit) was injected into an ear vein, and the time noted from injection until milk flowed by hand-milking one of the front glands (the delay time). The normally sudden stoppage of milk-flow was also timed, so that the duration of action of the hormone was recorded (the let-down time). In the light of the effects of adrenaline on the action of oxytocin (5), nothing was done which could in any way disturb the sow. Because of the difference in circulation time between the fore and the hind glands (2), the former were always used in our standard procedure.

It is interesting to note that, while sows familiar with the milking routine came voluntarily to the stall when released from the pen and paid little attention to the injection, yet in no case has a spontaneous release of milk to the milking procedure or to the injection of saline been recorded. The sow does not become conditioned to a routine which in some cases (in experiments not recorded here) occurred daily. Of course, the effect of the one machine milking each day would be completely inhibited by the effect of the twenty or so normal sucklings if we take a Pavlovian view of the reflex. In the work described it is fortunate that the spontaneous release of milk does not occur.

RESULTS

Table 1 sets out the data for six sows, three of which were milked frequently during the lactation. Table 2 gives the weekly body weights of the sows in question.

Table 1. *The delay time and let-down time for six sows when given 0·5 unit oxytocin*

Sow	Days in milk	Delay time (sec.)	Let-down time (sec.)
Rona	8	16·3	30·4
	10	15·1	29·4
	29	14·1	23·9
	36	17·3	24·7
	38	14·7	25·5
	43	16·8	18·9
	45	18·2	31·1
	56	21·4	23·5
Averages for sow		**16·7**	**25·9**
Rebecca	11	14·2	30·4
	14	15·5	19·5
	41	12·0	23·4
	42	14·0	24·0
	46	13·8	19·4
	48	16·1	41·1
	49	16·5	23·7
	54	16·8	25·5
Averages for sow		**14·9**	**25·9**
June	9	15·5	20·0
	11	14·2	34·1
	30	14·3	25·2
	33	13·5	37·7
	37	15·0	31·0
	39	14·1	24·0
	40	12·7	23·7
	44	13·6	23·1
	46	14·4	27·0
Averages for sow		**14·1**	**27·3**
Chrissie	10	15·2	25·2
	12	13·7	39·7
Averages for sow		**14·5**	**37·5**
Pauline	16	12·8	32·3
	34	13·8	27·1
	36	16·5	32·1
Averages for sow		**14·4**	**30·5**
Flika	27	12·7	29·4
	37	15·7	23·0
	48	13·8	28·7
Averages for sow		**14·1**	**27·0**
	Group averages	**15·0**	**27·5**

Table 2. *Weights of sows used in oxytocin experiments*

	Days in milk							
	7	14	21	28	35	42	49	56
Sow	Weight (lb.)							
Rona	361	350	340	341	319	326	311	312
Rebecca	410	388	396	379	363	366	362	357
June	377	372	362	353	342	342	345	345
Chrissie	306	300	296	284	280	266	271	265
Pauline	367	371	335	319	320	321	330	332
Flika	374	362	347	337	333	323	323	299

DISCUSSION

Neither circulation time nor milk let-down time showed any trends throughout lactation. The former was a more uniform property than the latter, though both showed a reasonable degree of uniformity in comparison with many other physiological 'constants'. It is interesting to compare the above circulation times with those recorded by Vercruysse, Peeters & Coussens (6) for the cow. The average figure for thirteen cows was 19·6 sec., with a range from 18 to 22. The average for the sows was 15·0 sec., with a range from 12·0 to 21·4.

The writer wishes to express his indebtedness to Mr D. M. Smith and Mr E. D. O'Reilly of this Station for their assistance in this work. Many of the data used are from experiments carried out in collaboration with Dr C. W. Turner and published jointly elsewhere.

REFERENCES

(1) FOLLEY, S. J. (1947). *Brit. med. Bull.* **5**, 1102.
(2) WHITTLESTONE, W. G. (1952). *J. Endocrinol.* (in the Press).
(3) WHITTLESTONE, W. G., BASSETT, E. G. & TURNER, C. W. (1952). *Proc. Soc. exp. Biol., N.Y.*, **80**, 191.
(4) BRAUDE, R. & MITCHELL, K. G. (1950). *Nature, Lond.*, **165**, 932.
(5) WHITTLESTONE, W. G. *J. Endocrinol.* (in the Press).
(6) VERCRUYSSE, R., PEETERS, G. & COUSSENS, R. (1950). *Arch. int. Pharmacodyn.* **82**, 93.

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637.124

495. THE EFFECT OF PREPARTUM MILKING ON THE COMPOSITION OF THE PREPARTUM AND POSTPARTUM SECRETIONS OF THE COW

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(With 2 Figures)

The increasing practice of milking cows before they calve has emphasized the need for a study of the changes that occur in the detailed composition of the secretion that is obtained during the prepartum period. This is especially desirable if the mechanism of the secretion of colostrum is to be understood. The general characteristics of colostrum have long been known; namely, the high percentage of total solids, protein (especially globulin) and ash, the low percentage of lactose and the variable fat percentage. In particular, the fat is characterized by lower Reichert and saponification values and a higher iodine value and melting-point than normal butter fat. Among earlier workers, Eckles & Palmer in 1916 (1), in addition to their study of these details of composition, and of the high carotene content of colostrum, found that no secretion of a colostral nature was produced when cows were milked throughout their normal dry period.

Woodman & Hammond in 1923 (2) analysed the secretions obtained from pregnant heifers and found that after about the fifth month of pregnancy a honey-like secretion was produced, containing about 40% of total solids and 35% of globulin, whereas previously the gland had elaborated a small amount of secretion containing the constituents of normal milk. This honey-like secretion was highly coloured and contained an appreciable amount of fat, which differed in composition from normal butterfat. These workers concluded that the formation of colostrum is not essentially connected with the period of parturition, and that in heifers colostrum is a mixture of true milk with the secretion formed during the fifth and sixth months of pregnancy. They considered that the occurrence of the honey-like secretion would not be noticed with the lactating pregnant cow owing to dilution brought about by milk secretion.

Asdell (3) milked a heifer at intervals throughout pregnancy and confirmed the results of Woodman & Hammond. After the time of appearance of the honey-like secretion, the percentage of globulin and albumin in the secretion decreased and that of lactose increased. Like Eckles & Palmer (1), he found an increase in the percentage of total protein, casein and globulin during the last three weeks of pregnancy, but these percentages decreased before parturition. He suggested that the globulin was either a continuous product of the growing cells of the alveoli or an excretion of the cell produced at the time of the change in metabolism from that of growth to that of milk secretion. Since he was unable to find any data that showed an increase in the amount of globulin produced by a lactating cow during the later stages of pregnancy, when mammary growth is taking place, he concluded that the globulin was present in the mammary ducts as a result of a change by the mammary cells from growth to secretion.

In America, Ragsdale, Weber & Turner (4) found that cows that had previously lactated also produced the honey-like secretion, and that pre-milking resulted in a secretion after calving that was similar in composition to that of normal milk. The carotene content of the prepartum secretion has been studied by Keyes (5), who showed an increase from the start of pre-milking to the day of calving, followed by a decrease to the fourth day after calving.

Vanlandingham, Weakley, Ackerman & Hyatt (6) found a great variation in the composition of the postpartum secretion from Ayrshire first-calf heifers that had been pre-milked for 10 days. Some heifers were stimulated into milk production whereas others were not. The percentage of total nitrogen and the percentage of the total present as non-casein nitrogen on the day of parturition was dependent on the total amount of prepartum secretion produced and the level of production at parturition, but was not necessarily related to the number of days prepartum on which milking had been carried out.

Eaton, Johnson, Spielman, Matterson & Nezvesky (7) confirmed the findings of earlier workers on the effect of prepartum milking on the percentages of protein, ash and lactose in the postpartum secretion. In addition, they showed that pre-milking resulted in a postpartum secretion of a lower specific gravity and containing less carotenoids and vitamin A, per 100 ml. of secretion and per g. of fat, than normal colostrum. During the period of prepartum milking they found, unlike Keyes (5), a negative trend in the carotene and vitamin A content of the secretion. This negative trend was temporarily arrested by parturition. Paul, Kalyanakrishnan, Lazarus, Anantakrishnan & Sen (8) confirmed these findings and also showed that during pre-milking the Reichert and saponification values of the fat increased while the iodine value was lowered. Parturition affected these values, causing a reversal of the trends, while after parturition the prepartum trends continued. This is in agreement with Krukovsky (9), who showed that carotenoids are associated with the fraction of fat having the highest iodine value. Paul *et al.* (8) also showed that the fat obtained postpartum from the half of an udder which had not been pre-milked was more comparable to the fat produced postpartum by the other half of the same udder, which had been pre-milked, rather than to the fat produced by it at the start of pre-milking.

Hill, Widdowson & Maggs (10) found the rate of change of the composition of prepartum secretion was less than that from normal colostrum to milk. They found that the postpartum secretion was usually intermediate between colostrum and normal milk, the composition depending partly upon the length of time that pre-milking had been practised.

From the above review it is apparent that while the effects of prepartum milking on the main constituents of colostrum had been established, further information was needed on the effect of parturition on the composition of the secretion, on the time of appearance of the globulin, and on the trends of other nitrogenous and ash components, and for this reason the following investigation was carried out.

EXPERIMENTAL

Cows and treatment

During the autumn and winter of 1949, eleven Shorthorn cows from the Institute herd were milked by hand twice daily starting on the 14th day before calving was expected: the mean period of pre-milking was 16 ± 1 days. The cows were 'steamed up' for a mean

period of 37 ± 1 days before calving with a compound concentrate (National Cattle Food no. 1, balanced for milk production), starting at 4 lb. daily and gradually increasing to 8 lb. daily for the last fortnight. The total amount secreted prepartum by the eleven cows varied from 1 to 96 l. per cow. The daily yields are shown in Fig. 1. All the cows calved normally except one which suffered from an everted uterus and milk fever.

The volume of the secretion obtained from these cows at each milking prepartum and for 4 days postpartum was measured, and daily samples of bulked evening and morning secretion were stored at $-25°$ C. for analysis. To cover the prepartum milking period, six to nine of the daily samples from each of eight of these cows were analysed, and for the postpartum period, four samples; their mean total prepartum yield being 39·75 l.

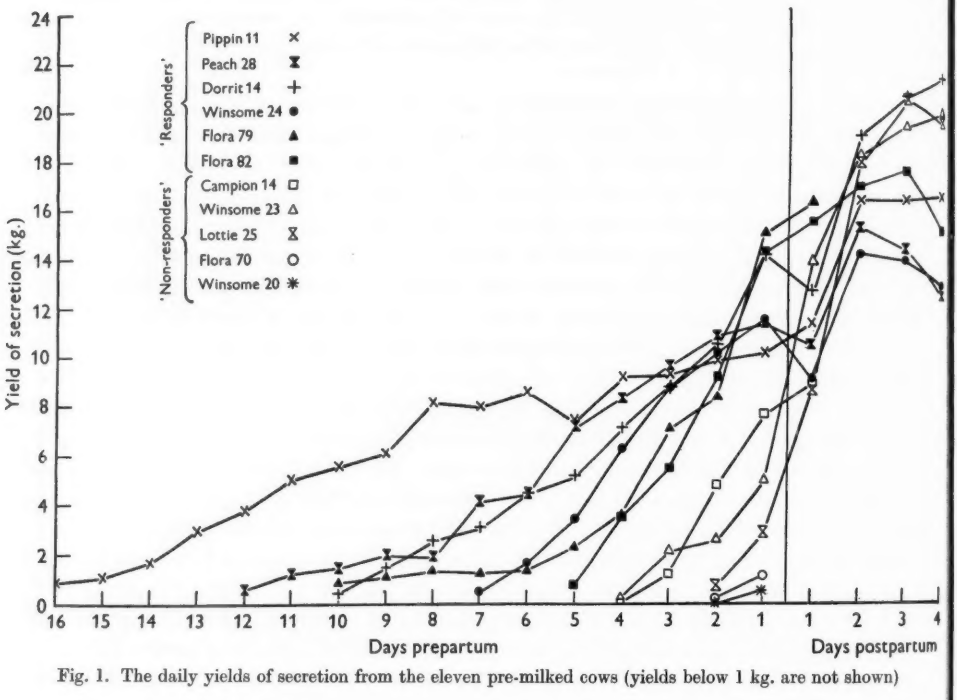

Fig. 1. The daily yields of secretion from the eleven pre-milked cows (yields below 1 kg. are not shown)

with a range from 3 to 96 l. For comparison with the composition of the pre- and post-partum secretion of these eight pre-milked cows, five samples of Shorthorn colostrum, each representing the total secretion obtained from the two milkings within the first 24 hr. after calving were also analysed.

Analytical methods

Fat was determined by the Gerber method (British Standards Institution (11)); total solids by the gravimetric technique of Golding (12); protein by the Kjeldahl method, the value for nitrogen being multiplied by the factor 6·38; lactose by a modification of the volumetric chloramine-T method of Hinton & Macara (13); ash by incineration in an electric muffle furnace at a temperature not exceeding 550° C.; calcium by precipitation

as oxalate from a solution of the ash, and titration with permanganate; phosphorus by precipitation as ammonium phosphomolybdate from a solution of the ash and titration with alkali; chloride by the wet digestion method of Davies (14); and the protein distribution and non-protein nitrogen (N.P.N.) by suitable adaptations of the methods of Rowland (15).

For the measurement of vitamin A and carotenoids, the milk-fat was extracted by a modification of the method of Olson, Hegsted & Peterson (16) and the fat saponified. A suitable dilution of the non-saponifiable residue in chloroform was used for the determination of vitamin A using the antimony trichloride reaction. The chloroform solution was also used for the measurement of carotenoids. The absorption measurements were made in the photoelectric spectrophotometer of Thompson (17).

Definitions

Normal colostrum is the secretion obtained in the first 24 hr. after calving from cows not pre-milked.

Postpartum secretion is the secretion obtained in the first 24 hr. after calving from pre-milked cows.

Day of initiation of milk secretion is the day, before and sometimes well before calving, on which the rapid increase in yield occurs.

RESULTS

The composition of the earliest sample prepartum for the eight cows is given in Table 1.

Table 2 shows the mean composition of the secretion obtained during the first 24 hr. after calving from the eight pre-milked cows, and also that of the normal colostrum collected during the same period postpartum from five cows. As the volume of secretion removed from the udder before calving influences the composition of the postpartum secretion, the five pre-milked cows that had given over 30 l. before calving were grouped together as 'responders'. A comparison was then made between the composition of the normal colostrum and the composition of the postpartum secretion from (*a*) the five 'responders' and (*b*) all the eight pre-milked cows. Statistical significance of differences between the mean values of the various constituents, as determined by the '*t*' test, are given at the bottom of the table, *P* values below 0·05 being regarded as significant.

The weights of the various constituents produced daily by each of the eight cows were calculated. Likewise, the total daily production of vitamin A and carotenoids were calculated from the weight of fat. It was observed that, of the weights of the various constituents determined, the weight of colostral globulin produced daily by each of the eight cows, unlike that of the other constituents, was characterized by an increase up to a maximum on a certain day before calving, and then a decline. As the interval between the peak production of globulin and calving varied widely between the cows, it was decided to co-ordinate all the analytical data for the eight cows making their peak production of globulin coincide and recording the other data from this base-line. Interpolations were made for each day on which analyses were not done, and daily means, for a period on each side of the globulin peak, were then calculated for all the constituents. The mean period between this peak production of globulin and parturition was $5 \pm 1{\cdot}5$ days. Fig. 2 (*c*)–(*o*) gives the daily means for each of the constituents as percentages and as weights, and the mean day of parturition. Fig. 2 shows the yield of secretion and the

Table 1. *Composition of the earliest sample prepartum from the eight pre-milked cows*

Constituent	Range	Mean
Total solids (%)	23·3 –37·1	30·3
Fat (%)	0·5 – 4·2	1·8*
Solids-not-fat (%)	19·1 –35·9	27·5*
Lactose (%)	0·4 – 2·1	1·3
Protein (%)	16·2 –31·8	25·9
Ash (%)	0·52– 1·08	0·74
Calcium (mg./100 g.)	16– 191	65
Phosphorus (mg./100 g.)	7– 177	57
Chloride (mg./100 g.)	187– 288	237
Casein-N (mg./100 g.)	196–1257	687
Albumin-N (mg./100 g.)	278– 665	445
Globulin–N (mg./100 g.)	1163–3718	2718
Proteose peptone-N (mg./100 g.)	40– 288	136
Non-protein-N (mg./100 g.)	19– 146	68
Vitamin A (i.u./g. fat)	87– 474	253
Carotenoids (μg./g. fat)	48– 175	119

* Samples from only six cows were available for these constituents.

Table 2. *Mean values with their standard errors for various constituents of normal colostrum and of the postpartum secretion from pre-milked cows*

											Nitrogen fractions						
	Yield pre-partum (l.)	Total solids (%)	Fat (%)	Solids-not-fat (%)	Lactose (%)	Protein (%)	Ash (%)	Ca (mg./100 g.)	P (mg./100 g.)	Cl (mg./100 g.)	Casein (mg./100 g.)	Albumin (mg./100 g.)	Globulin (mg./100 g.)	Proteose and peptone (mg./100 g.)	Non-protein (mg./100 g.)	Vitamin A (i.u./g.)	Carotenoids (μg./g.)
Postpartum secretion from all pre-milked cows	39·75 ±11·03	14·70 ±0·66	3·74 ±0·30	10·96 ±0·84	4·13 ±0·16	5·21 ±0·94	0·90 ±0·22	147 ±5	133 ±9	109 ±7	502 ±45	110 ±28	119 ±80	30 ±2	56 ±4	72 ±19	17 ±4
Postpartum secretion from 'responders'	57·75 ±10·98	14·27 ±0·18	4·15 ±0·34	10·12 ±0·40	4·38 ±0·12	4·03 ±0·12	0·87 ±0·14	152 ±5	121 ±4	99 ±7	455 ±14	71 ±21	25 ±3	29 ±3	50 ±3	46 ±12	12 ±3
Normal colostrum		22·17 ±1·37	3·63 ±0·79	18·54 ±1·06	3·10 ±0·26	14·25 ±1·25	0·97 ±0·02	165 ±7	151 4	147 ±10	819 ±59	229 ±42	1070 ±138	61 ±5	55 ±3	Estimations not done	
		Statistical significance of differences between the mean values for normal colostrum and those for the postpartum secretion from both 'responders' and all pre-milked cows															
Normal colostrum and postpartum secretion from all pre-milked cows (*P*)		<0·001	n.s.	<0·001	<0·01	<0·001	n.s.	n.s.	n.s.	<0·01	<0·01	<0·05	<0·001	<0·001	n.s.		
Normal colostrum and postpartum secretion from the 'responders' (*P*)		<0·001	n.s.	<0·001	<0·01	<0·001	<0·01	n.s.	<0·001	<0·01	<0·001	<0·02	<0·001	<0·001	n.s.		

weights of many constituents to fall on the second day postpartum, and to continue their previous trends thereafter. This apparent temporary fall does not represent a real change in weight but is due solely to the method of co-ordinating the analytical data. There were two cows, for which the calving dates differed greatly from the mean time of calving and the yields of secretion and weights of constituents were very high, with the result that their data could not be included after the first day postpartum, and thus the means of the yield of secretion and of the weights of constituents for the remaining cows fell sharply on the second day postpartum.

The changes in the daily yield of the cows are shown in Fig. 2 (*a*) and (*b*); (*a*) gives the directly observed yields, and (*b*) each day's relative yield as a percentage of the previous day's yield with the weight of globulin-N on the same graph to show the relation between these two factors. The relative yield in Fig. 2 (*b*) is based on a moving average of the values for three consecutive days to remove minor day-to-day fluctuations.

DISCUSSION

Composition of the earliest prepartum secretion and normal colostrum

It is clear from the data of Tables 1 and 2 that in spite of the large range in concentration of the various constituents observed in the earliest prepartum secretion, the concentrations of those constituents which are characteristically high in normal colostrum are even higher in this early secretion. Thus, the concentration of total solids, solids-not-fat, total protein, albumin, globulin and chloride are higher in the earliest prepartum secretion, whereas the concentration of lactose is lower. The concentrations of fat, ash, calcium, phosphorus, casein, proteose-peptone and N.P.N. in the earliest prepartum secretion did not appear to differ from their concentration in normal colostrum.

Trends in the composition of the secretion during prepartum milking

The concentrations of solids-not-fat, total protein, albumin, globulin, proteose-peptone and chloride fell sharply after the initiation of milk secretion, particularly the content of globulin. The concentration of fat (Mukherjee, Swaminathan & Viswonath (18)), and also that of lactose, increased throughout the prepartum period, the greatest increase in lactose occurring at the time of the greatest increase in yield. This is in agreement with the findings of Taylor & Husband (19), who suggested that the concentration of lactose is related to the rate of milk secretion. The concentrations of calcium and of phosphorus steadily increased, and at about the same rate, up to the time of the peak in the weight of globulin, but thereafter the concentration of calcium remained nearly constant, while that of phosphorus fell. The concentration of casein increased to a maximum at the time of initiation of milk secretion and then fell. This confirms the view of Hammond (20) that in the cow there appears proportionately more casein than lactose in the early stages of the evolution of milk secretion. The concentrations of carotenoids and vitamin A showed trends similar to that of casein, but there was a slight increase in both values at the same time after calving as the slight increase in weight of globulin occurred. In contrast to that of the other constituents, the concentration of N.P.N. remained fairly constant during the period of prepartum milking.

Thus the composition of the secretion during the period of prepartum milking changed progressively towards that of milk, but the rate of change varied considerably. It was rapid for those animals which readily responded to prepartum milking with increasing

from the 'responders' (*P*)

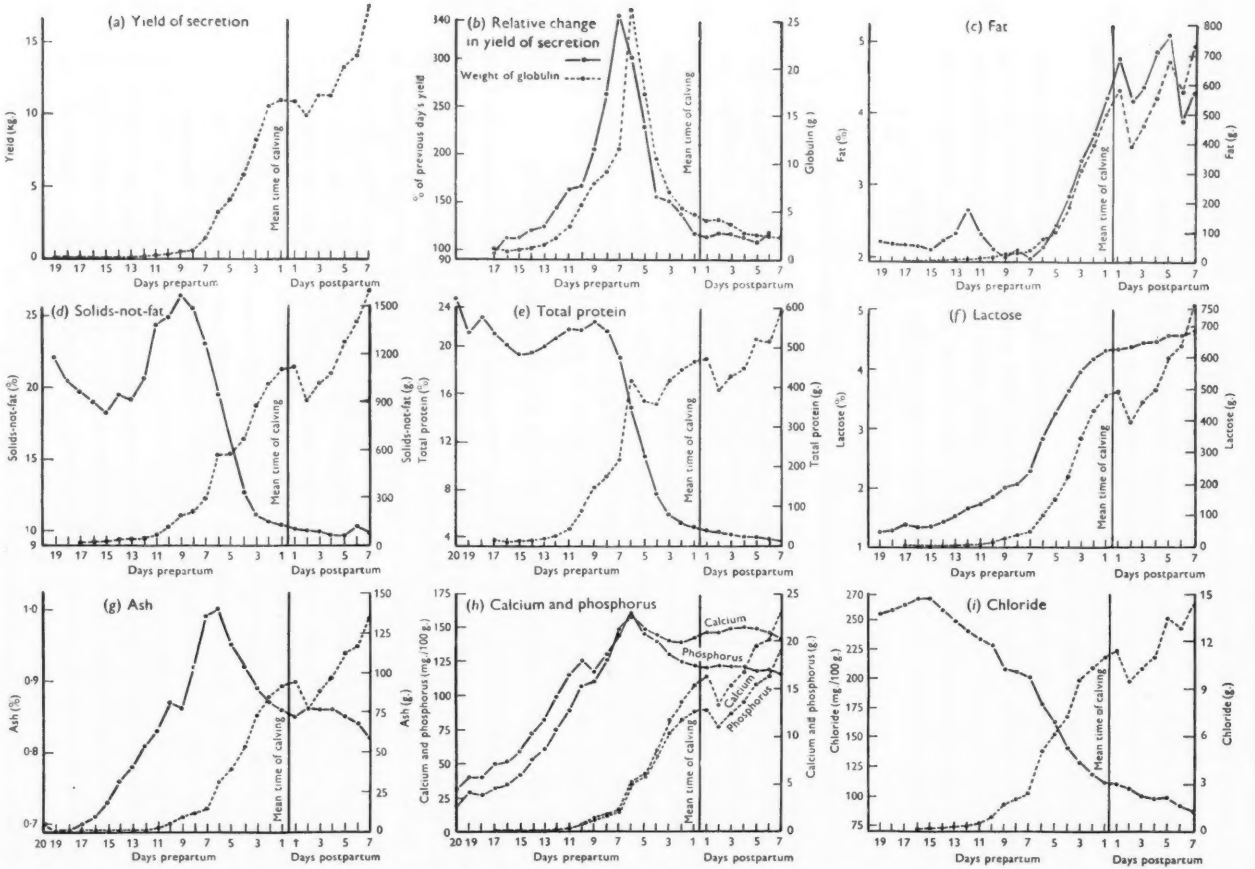

Fig. 2 (*a–i*). The effect of prepartum milking on the yield and composition of the prepartum and postpartum secretions of eight Shorthorn cows. The figures show the changes in composition around the time of peak globulin production, and the corresponding mean time of calving (6 days later). (*a*) yield of secretion, (*b*) the relative change in yield of secretion, and the weight of globulin, (*c*) fat, (*d*) solids-not-fat, (*e*) total protein, (*f*) lactose, (*g*) ash, (*h*) calcium and phosphorus, (*i*) chloride. Percentage ——·——, weight – – – – –.

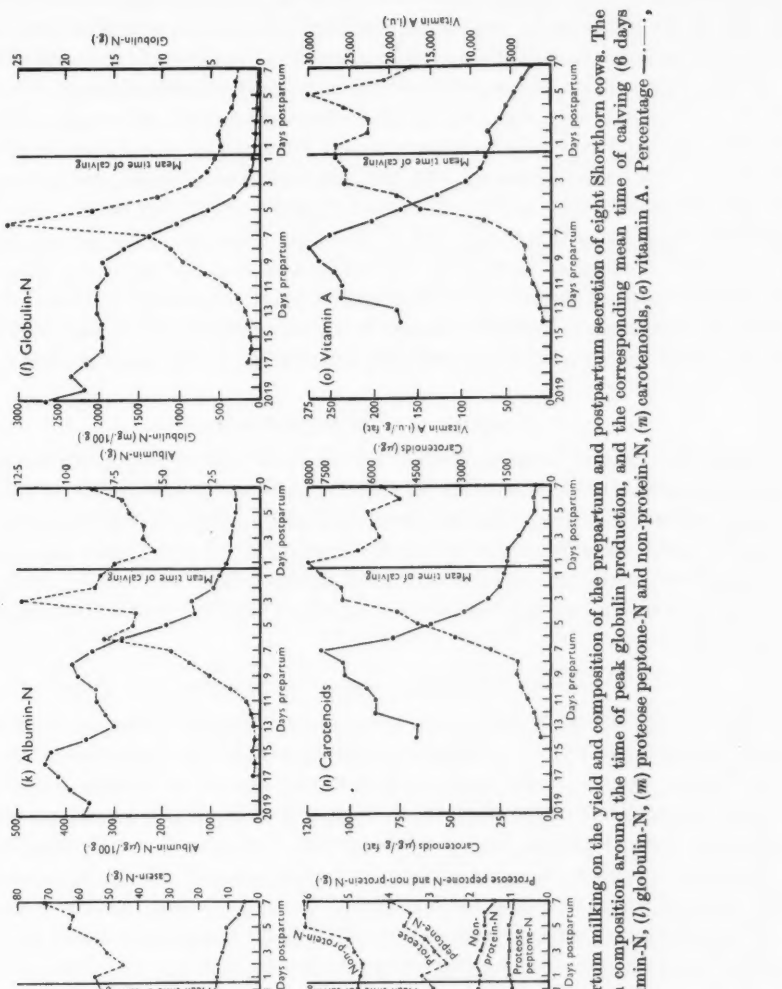

Fig. 2. (*j–o*). The effect of prepartum milking on the yield and composition of the prepartum and postpartum secretion of eight Shorthorn cows. The figures show the changes in composition around the time of peak globulin production, and the corresponding mean time of calving (6 days later). (*j*) casein-N, (*k*) albumin-N, (*l*) globulin-N, (*m*) proteose peptone-N and non-protein-N, (*n*) carotenoids, (*o*) vitamin A. Percentage —·—·—, weight — — — —.

yields; they were producing for a few days before calving a true milk of slightly high solids-not-fat content, such as is produced after the colostral stage and for the first week or two of the lactation by cows not pre-milked. For the animals that gave a small or negligible response in yield, the secretion was still of the colostral type until after calving.

With the exception of the weight of globulin which showed a sharp peak before the cows calved and a very slight rise and fall after they calved, the weights of the various constituents produced daily by the eight cows increased during the period of prepartum milking as the yields increased. The globulin peak was reflected also in a peak in the weight of total protein, and it will be seen from Fig. 2 (*b*) that the peak production of globulin coincided within a day with the maximum increase in the daily yield.

The total amount of carotenoids and vitamin A increased up to the time of calving and then tended to decrease. The irregularities found postpartum in these totals are due to the normal large individual variation in the values for these components affecting the mean, when the number of observations contributing to the mean is altered.

Composition of the postpartum secretion

From the trends in composition of the secretion during prepartum milking, it was evident that the composition of the postpartum secretion, especially from the 'responders', would differ widely from that of normal colostrum. This was confirmed by the significantly lower concentration of all the constituents in the postpartum secretion from the 'responders', with the exception of that of lactose which was higher in the postpartum secretion, and that of fat, calcium and N.P.N., which did not differ between the two secretions.

The relationship between carotenoids, vitamin A and globulin

The earlier findings of other workers have established the following facts: (1) Normal colostrum is characterized by a high concentration of globulin, carotenoids and vitamin A. (2) Plasma vitamin A and carotenoids decrease from about 1 week before calving to within 3 days postpartum, the level subsequently rising, but not reaching normal levels for about 4 weeks (Sutton, Kaeser & Soldner (21)) Eaton, Johnson, Helmboldt, Spielman, Matterson, Jungherr, Kramer & Slate (22) found, however, no significant difference for the time of maximum depression of plasma carotenoids and vitamin A between cows milked prepartum and only postpartum (3) The fat found in the honey-like prepartum secretions is different from normal butterfat (Woodman & Hammond (2)) In prepartum-milked cows, more of this abnormal fat appears after calving when the negative trends in the concentration of globulin, carotenoids and vitamin A are temporarily arrested (Mukherjee *et al.* (18); Vanlandingham *et al.* (6); Paul *et al.* (8)). Moreover, Mercer, Eaton, Johnson, Spielman, Plastridge, Matterson & Nezvesky (23) failed to influence the total production of carotenoids and vitamin A by interruption of milking for 10 days and suggested that under the conditions of their experiment the secretion of vitamin A might be independent of that of fat.

In view of these facts it seemed possible that both the carotenoids and vitamin A were associated with two different fractions of colostrum; part being associated with normal butterfat and part, in very much higher concentration, with some lipid appearing in the udder at about the same time as the globulin. The presence of such a lipid could account for the changes in the fat constants.

In an attempt to justify this hypothesis, the relevant results for the eight cows were pooled, and multiple regression analyses of the daily concentration of carotenoids (μg./g.) and vitamin A (i.u./g.) on both the daily weight of fat and daily weight of globulin-N were done. The values for weight of fat were transformed to the log scale, because of the great variation in level of production from the start of pre-milking to parturition. The partial correlation coefficients were as follows.

Concentration of vitamin A on weight of fat	−0·53***
Concentration of vitamin A on weight of globulin	+0·50***
Concentration of carotenoids on weight of fat	−0·67***
Concentration of carotenoids on weight of globulin	+0·41**

** Significant when $P=0{\cdot}01$. *** Significant when $P=0{\cdot}001$. Degrees of freedom = 54.

These data show that for the eight pre-milked cows, the daily concentration of vitamin A and carotenoids was negatively correlated with the daily weight of fat and positively correlated with the daily weight of globulin.

General

Apart from confirming the findings of earlier workers concerning the effects of prepartum milking on the main constituents of the prepartum and postpartum secretions, our data have given information on the effect of this practice on the trends of calcium, phosphorus, chloride and the various nitrogen fractions before and after calving.

Of particular interest is the relationship, mentioned earlier, and shown in Fig. 2 (*b*), between the daily weight of globulin produced and the maximum increase in milk yield. This raises two possibilities: (1) the globulin passes from the blood stream at this time, as a result of some hormonal effect which is also responsible for the initiation of milk secretion, or (2) the globulin actually accumulates in the udder for some time before the beginning of milk secretion, and owing to its physical properties is not eluted until there is a very rapid flow of milk. Existing knowledge does not permit of a definite decision as to which of these two possibilities is correct, but the former would appear to be more likely. It is of interest that even when cows are milked throughout their normal dry period (Eckles & Palmer (1), Asdell (3)), with the result that there is no normal colostrum production, there is nevertheless an increase in the percentage of soluble proteins at the time of calving. This suggests that the maximum production of globulin occurs at this time, and that in cows, as distinct from in first-calf heifers (Woodman & Hammond (2)), little globulin accumulates in the udder earlier in the dry period. In this connexion, the work of Dicks, Eaton & Carpenter (24) should be mentioned. They were unable to influence the total production of globulin by interruption of milking for 10 days during normal lactation. This finding adds further weight to the theory that the production of globulin, in large quantities, is dependent on the physiological state of the cow during the last stages of pregnancy.

Further support for this theory comes from Woodman & Hammond (2), who considered that the high globulin content of the honey-like secretion obtained from heifers after the fifth month of pregnancy could not result solely from the filtration of globulin from the blood during the inactivity of the mammary gland. They quoted Oswald (25), who found that during inflammation the permeability of the capillaries is increased, and suggested it was possible that specific hormones developed in pregnancy might affect the capillaries similarly, thus allowing large molecules like those of proteins to pass through. There is

little doubt that a change in the hormone balance of the heifer occurs at the fifth month of pregnancy, since after this time, the alveoli of the udder begin to develop, the foetus begins its stage of greatest growth, and milk, in some quantity, is produced in cases of abortion (Woodman & Hammond (2), Asdell (3), Hammond (26)). Hechter, Krohn & Harris (27) found that oestrogens affect the permeability of the capillary blood vessels of the uterus and vagina, and it seems possible that this might occur in the udder of the cow, as distinct from the heifer, at the time of milk secretion. It is believed that pituitary lactogen secretion is governed primarily by the level of active circulating oestrogen within the body (Meites & Turner (28)), and it is also known that the oestrogens present in colostrum occcur in small quantities in colostral fat as well as in separated colostrum (Pope & Roy (29)). This latter fact is of particular interest in view of the close association between the globulin and abnormal fat found in the honey-like secretion of heifers (Woodman & Hammond (2)). It seems probable that the actual act of calving results in a slightly increased output of globulin. A slight rise after calving in weight of globulin occurred in our experiment, and an abrupt rise in percentage after calving was found by Mukherjee *et al.* (18), when cows were pre-milked for a few days only. Against the possibility of some sudden hormonal effect on the permeability of the capillaries and the resulting sudden passage of globulins into the gland soon after the initiation of milk secretion, there is the conclusion by Smith & Greene (30) that colostral globulin, though very closely related to the T-component of bovine plasma, is not identical with it.

It seems that part of the total vitamin A and carotenoids appear in the udder at the same time as the globulin. Further work is, therefore, required to establish whether a peak production of carotenoids and vitamin A, coincident with that of globulin, occurs simultaneously with a depression in plasma carotenoids and vitamin A; and to find whether a depression in plasma γ- or T-globulin occurs at the time of maximum production of globulin by the udder, and is positively correlated with this depression of carotenoids and vitamin A. A depression in a protein fraction of the sera from ewes in the latter stage of pregnancy has been found by Dalgarno, Godden & McCarthy (31), who suggest that it was associated with colostrum formation.

The variability in total prepartum yield found in our experiment agrees with that observed by Vanlandingham *et al.* (6), who found total yields varying from 2 to 142 lb. for eight first-calf Ayrshire heifers pre-milked for 10 days. They suggested that prepartum milking might stimulate the production of lactogenic hormone, and pointed out that cows vary in response to this stimulus. It was also evident from our experiment that there was a secondary stimulation of milk production after parturition due possibly to the removal of some inhibitory factor, possibly arising from products of the placenta. The removal of such a factor might also account for the slight rise and fall in globulin at this time.

SUMMARY

Eleven Shorthorn cows were milked for 14 days before their expected calving date. The total amount of prepartum secretion varied, between cows, from 1 to 96 l.

Determinations of total solids, fat, solids-not-fat, lactose, total protein, ash, calcium, phosphorus, chloride, casein, albumin, globulin, proteose-peptone, N.P.N., vitamin A and carotenoids were made regularly on the secretions of eight of these cows during the prepartum period, and for 4 days postpartum; and, for comparison, on five samples of Short-

horn colostrum each representing the total secretion obtained from the two milkings within the first 24 hr. after calving.

The concentrations of those constituents which are characteristically high in normal colostrum are even higher in the earliest prepartum secretion. The composition of this secretion changed progressively during the period of prepartum milking towards that of milk, but the rate of change varied considerably. It was rapid for those animals which readily responded to prepartum milking with increasing yields; but for those that gave only a small response in yield, a secretion of the colostral type persisted until after calving.

In comparison with the composition of normal colostrum, the concentration of solids-not-fat, protein, ash, phosphorus, chloride, casein, albumin, globulin and proteose-peptone was significantly lower in the postpartum secretion of pre-milked cows, and the concentration of lactose significantly higher. The concentration of fat, calcium and N.P.N. in the two secretions did not differ. The actual weights of the various constituents produced increased daily during the period of prepartum milking as the yields increased, with the exception of the weight of globulin which showed a definite maximum at the time when a rapid increase in milk yield was initiated.

The daily concentration of vitamin A and carotenoids during the period of prepartum milking was negatively correlated with the daily weight of fat and positively correlated with the daily weight of globulin produced. This relationship between globulin, vitamin A and carotenoids is discussed, and a theory of the origin of these substances in colostrum is formulated.

We are indebted to Miss R. Thorner, Miss P. Turner, Miss M. Vardy and Mr A. Wagstaff for their valuable help with the large number of analyses, and this is gratefully acknowledged.

REFERENCES

(1) ECKLES, C. H. & PALMER, L. S. (1916). *J. biol. Chem.* **27**, 313.
(2) WOODMAN, H. E. & HAMMOND, J. (1923). *J. agric. Sci.* **13**, 180.
(3) ASDELL, S. A. (1925). *J. agric. Sci.* **15**, 358.
(4) RAGSDALE, A. C., WEBER, C. W. & TURNER, C. W. (1929). *Bull. Mo agric. Exp. Sta.* no. 272, p. 44.
(5) KEYES, E. A. (1944). *Milk Dlr*, **33**, 12, 50.
(6) VANLANDINGHAM, A. H., WEAKLEY, C. E. (Jr.), ACKERMAN, R. A. & HYATT, G. (Jr.) (1949). *J. Dairy Sci.* **32**, 559.
(7) EATON, H. D., JOHNSON, R. E., SPIELMAN, A. A., MATTERSON, L. D. & NEZVESKY, L. (1949). *J. Dairy Sci.* **32**, 919.
(8) PAUL, T. M., KALYANAKRISHNAN, M. G., LAZARUS, A. J., ANANTAKRISHNAN, C. P. & SEN, K. C. (1950). *Indian J. Dairy Sci.* **3**, 112.
(9) KRUKOVSKY, V. N. (1942). *J. Dairy Sci.* **25**, 297.
(10) HILL, R., WIDDOWSON, R. W. & MAGGS, B. E. (1950). Mimeograph, Roy. Agric. College, Cirencester.
(11) British Standards Institution (1936). Specification no. 696, part 2, p. 9.
(12) GOLDING, J. (1934). *Analyst*, **59**, 468.
(13) HINTON, C. L. & MACARA, T. (1927). *Analyst*, **52**, 668.
(14) DAVIES, W. L. (1932). *Analyst*, **57**, 79.
(15) ROWLAND, S. J. (1938). *J. Dairy Res.* **9**, 30–46.
(16) OLSON, F. R., HEGSTED, D. M. & PETERSON, W. H. (1939). *J. Dairy Sci.* **22**, 63.
(17) THOMPSON, S. Y. (1949). *Brit. J. Nutrit.* **3**, 43.
(18) MUKHERJEE, S. K., SWAMINATHAN, K. & VISWONATH, B. (1944). *Indian J. vet. Sci.* **14**, 232.
(19) TAYLOR, W. & HUSBAND, A. D. (1922). *J. agric. Sci.* **12**, 111.
(20) HAMMOND, J. (1936). *Vet. Rec.* **48**, 519.

(21) Sutton, T. S., Kaeser, H. E. & Soldner, P. A. (1945). *J. Dairy Sci.* **28**, 933.
(22) Eaton, H. D., Johnson, R. E., Helmboldt, C. F., Spielman, A. A., Matterson, L. D., Jungherr, E. L., Kramer, J. H. & Slate, R. J. (1949). *J. Dairy Sci.* **32**, 870.
(23) Mercer, D. N., Eaton, H. D., Johnson, R. E., Spielman, A. A., Plastridge, W. N., Matterson, L. D. & Nezvesky, L. (1949). *J. Dairy Sci.* **32**, 977.
(24) Dicks, M. W., Eaton, H. D. & Carpenter, C. A. (1951). *J. Dairy Sci.* **34**, 52.
(25) Oswald (1910). *Z. exp. Path.* **8**, 226.
(26) Hammond, J. (1927). *Reproduction in the Cow.* Cambridge University Press.
(27) Hechter, O., Krohn, L. & Harris, J. (1942). *Endocrinolology*, **30**, 598.
(28) Meites, J. & Turner, C. W. (1948). *Res. Bull. Mo agric. Exp. Sta.* no. 415.
(29) Pope, G. S. & Roy, J. H. B. (1953). *Biochem. J.* To be published.
(30) Smith, E. L. & Greene, R. D. (1947). *J. biol. Chem.* **171**, 355.
(31) Dalgarno, A., Godden, W. & McCarthy, E. F. (1950). *Biochem. J.* **46**, 162.

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496. THE ASSOCIATIVE GROWTH OF *BACILLUS CEREUS* AND *STREPTOCOCCUS LACTIS* IN MILK

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INTRODUCTION

Bacillus cereus and its variants produce a characteristic type of spoilage in milk known as broken cream, which is quickly followed by sweet curdling (1). It has been shown that spores of the causative organism are present in most raw and pasteurized milk supplies (1). Although it is commonly reported that the fault appears in well-produced raw milk and in pasteurized milk, in our experience it is always distributors of pasteurized milk who receive complaints of broken cream.

The factors which affect the type of bacterial flora dominant in milk at the end-point of keeping quality tests are obviously complex. Soriano (2) recorded the predominance of *B. cereus* or *B. cereus* var. *albolactis* at the end-point of the clot-on-boiling (C.O.B.) test of samples of pasteurized milk incubated at 25° C., while Rowlands & Provan (3), in a study of milk pasteurized by the 'in-bottle' process found that *Streptococcus lactis* was dominant at the C.O.B. end-point in most of their samples held at 15·5° C. At the end-point of the 68% alcohol precipitation test on pasteurized milk held at 20° C., Egdell & Bird (4) found that in some cases streptococci and in others aerobic spore-formers predominated. On the other hand, Garvie & Rowlands (5), in a survey of the flora of raw milk samples held at 22° C. until the C.O.B. end-point, failed to isolate any spore-bearing aerobes. In eleven of the twelve samples they examined, spoilage appeared to be mainly due to the development of group N streptococci.

These observations indicate that it is pasteurized rather than raw milk which is liable to develop broken cream. It has been shown (6) that in pasteurized milk conditions are conducive to the development of *B. cereus*, whereas in raw milk the organism frequently fails to develop. This may be attributed to at least two causes. The spore-bearing aerobes in raw milk are generally outnumbered by acid-producing organisms which, unless they are reduced by pasteurization, develop rapidly and ultimately cause spoilage. Alternatively, as has been suggested by Davis (7), acid-forming organisms such as *Str. lactis* actually suppress the growth of *B. cereus* and thus prevent broken cream. Some preliminary experiments (6) have cast some doubt on Davis's statement, and the work described here was designed to investigate further both of the above possibilities.

EXPERIMENTAL

Two series of experiments were conducted. In the first series *B. cereus* and *Str. lactis* were inoculated into milk in varying proportions and incubated at a constant temperature. In the second group of experiments the level of inoculum was fixed and the temperature of storage was varied.

The effect of Bacillus cereus *and* Streptococcus lactis *together, upon the keeping quality of milk at* 22° C.

Milk from two heifers in mid-lactation was drawn aseptically into a sterile 1500 ml. flask. In the laboratory it was distributed in 50 ml. quantities in 8 × 1 in. heat-resistant glass tubes. The milk was pasteurized at 63° C. for 30 min., cooled and stored in the refrigerator until required.

One series of tubes was inoculated with the required number of each organism at 9 a.m. and a duplicate series was inoculated at 9 p.m., according to the method used by Garvie & Rowlands (8). The a.m. sample was inoculated from a fourth and the p.m. sample from a fifth transfer of the culture in dextrose Lemco broth (1% peptone, 1% Lemco, 0·5% NaCl, 1% dextrose). A preliminary trial with pure cultures of the selected *B. cereus* cultures showed that the keeping quality of milk inoculated with spores (and either pasteurized or not heat-treated) did not differ significantly from that of milk inoculated from a 24 hr. broth culture which presumably consisted mainly of vegetative cells.

After inoculation, the samples were mixed and 10 ml. of each transferred to a test-tube, 1 ml. of methylene-blue solution added and the tube incubated at 22° C. It was found that neither *B. cereus* nor *Str. lactis*, at the levels of inoculum used in these experiments, reduced methylene blue at 37·5° C. within 11 hr. and therefore observations for methylene-blue reduction were only made at 22° C. The remainder of each sample was incubated at 22° C. and examined for keeping quality by the 68% alcohol precipitation test and C.O.B. test.

The initial inoculum of each organism was estimated by poured plate counts on yeast dextrose agar (1% peptone, 1% Lemco, 0·5% NaCl, 0·5% dextrose, 0·3% yeastrel, 2% agar) of the appropriate dilution tubes from which the milk was inoculated. The colony count of each sample at end-point of methylene-blue reduction and at C.O.B. was made by the drop method of Miles & Misra (9) on the surface of yeast dextrose agar for *B. cereus* and yeast dextrose agar containing 0·1% thallium acetate for *Str. lactis*.

The effect of storage temperature on the associative growth of Bacillus cereus *and* Streptococcus lactis *in milk*

The experiments were based on those described by Stone (6). Milk was obtained as before but pasteurized in bulk and distributed immediately before use in 90 ml. quantities in 150 ml. flasks. The milk was inoculated with the required number of each organism from a fourth transfer of a culture in dextrose Lemco broth. After inoculation, one flask was stored at 5, one at 10 and one at 15° C. for 24 hr. At the end of this time the contents of each flask were distributed in 3 sterile 8 × 1 in. glass tubes, which were subsequently held at 15, 18 and 22° C. These subsamples were examined by the alcohol (68%) precipitation test after 30, 48, 51, 54, 72, 75, 78 and 96 hr., and observations for broken cream were made at the same time.

The initial inoculum of each organism was estimated from poured plate counts on the broth culture from which the milk was inoculated. Differential colony counts were made after 6 and 24 hr. from poured plates on yeast dextrose agar and yeast dextrose agar containing 0·1% thallium acetate. Colonies of *B. cereus* were sufficiently developed on yeast dextrose agar after 24 hr. incubation at 30° C. to be easily differentiated from those of *Str. lactis*.

Colony counts were made by the method of Miles & Misra after 30 hr., 48 hr. and again when the samples reached end-point of the alcohol precipitation test.

RESULTS AND DISCUSSION

The effect of Streptococcus lactis *and* Bacillus cereus *together upon keeping quality of milk at* 22° C.

Two strains of *Str. lactis* (35C.9 and 32C.15) were grown in turn with two strains of *B. cereus* (BC60 and BC127) and one of *B. cereus* var. *albolactis* (484) supplied by Miss Bird, N.A.A.S., Bristol.

In Table 1 are shown the initial proportions of the two organisms, the proportions at C.O.B. end-point, the time taken to reach C.O.B. end-point and observations for broken cream. It can be seen that in every case the *Streptococcus* greatly outnumbered the spore-former at the end-point of the C.O.B. test, whatever the initial proportion of the two organisms. Actual numbers of 35C.9 were in all cases greater than those of 32C.15 at C.O.B. end-point. It was found in preliminary experiments with pure cultures that 32C.15 grew more slowly than 35C.9, and the time taken for milk inoculated with 32C.15 to reach the C.O.B. end-point was longer, thus discounting the possibility that the spore-former had an inhibitory effect.

Each combination of *Streptococcus* and spore-former was examined at least twice, and for each sample there were subsamples prepared at both 9 a.m. and 9 p.m. In all, colony counts at the end-point of the C.O.B. test and broken cream were recorded on eighty samples and broken cream recorded on a further eighty. Broken cream was not observed in thirty-four samples, none of which had more spore-formers than *Streptococcus* in the milk initially and twenty-three had an initial ratio of streptococcus to spore-former of 100:1. Nine tubes in which broken cream was not observed had a colony count of *B. cereus* at the end-point of the C.O.B. test of less than 100,000, and only two tubes with a count of this order showed broken cream, which suggests that the *Streptococcus* developed very quickly and the milk reached end-point before the spore-former was present in sufficient numbers to cause spoilage.

From our observations it appears that if *B. cereus* is present in milk broken cream occurs, except in certain cases where the proportion of acid-producing organisms is so great as to cause spoilage before the spore-former has multiplied to an appreciable extent. Since it is unlikely that commercially pasteurized milk contains a high proportion of lactic streptococci to spore-formers and may even contain more spore-forming organisms than streptococci (10), it seems that the prevalence of broken cream in pasteurized milk may follow the simplification of the flora by heat.

The effect of storage temperature on the associative growth of Bacillus cereus *and* Streptococcus lactis *in milk*

Six experiments were carried out in which the two strains of *Str. lactis* were grown in turn with the three strains of *B. cereus* in milk stored under nine different combinations of temperature. Similar work with pure cultures of the spore-former had shown that levels of inoculum of the order of 1 spore per ml. gave rather erratic results, but it was considered that conditions simulating those found in practice would be obtained by using fairly low levels of inoculum. Therefore in these experiments the milk was inoculated with approximately 100 cells per ml. of *B. cereus* and 1 cell per ml. of *Str. lactis*.

Table 1

No. of culture and no. of cells inoculated/ml. milk		Proportion of spore-former: *streptococcus* at time of C.O.B. test end-point					Time of C.O.B. test end-point at 22° C.	Broken cream
Streptococcus	Spore-former	1: >100	1:100–50	1:50–10	1:10–1	1: <1		
35C.9	BC60							
10,000	10,000	+	.	.	.	.	27	Not recorded
100	10,000	+	.	.	.	.	30	Not recorded
1	10,000	.	.	+	.	.	30	Not recorded
10,000	100	+	.	.	.	.	33	Not recorded
100	100	.	+	.	.	.	36	Not recorded
1	100	.	+	.	.	.	33	Not recorded
10,000	1	+	.	.	.	.	33	Not recorded
100	1	+	.	.	.	.	36	Not recorded
1	1	.	.	+	.	.	42	Not recorded
35C.9	BC127							
10,000	10,000	.	+	.	.	.	18	±
100	10,000	.	.	+	.	.	27	+
1	10,000	.	.	+	.	.	30	+
10,000	100	+	.	.	.	.	24	±
100	100	+	.	.	.	.	30	+
1	100	.	+	.	.	.	30	+
10,000	1	+	.	.	.	.	24	±
1	1	+	.	.	.	.	33	+
35C.9	484							
10,000	10,000	+	.	.	.	.	33	+
100	10,000	.	+	.	.	.	36	+
1	10,000	.	+	.	.	.	33	+
10,000	100	+	.	.	.	.	33	−
100	100	.	.	+	.	.	36	+
1	100	.	.	+	.	.	36	+
10,000	1	+	.	.	.	.	33	−
1	1	+	.	.	.	.	39	−
32C.15	BC60							
10,000	10,000	.	.	+	.	.	30	+
100	10,000	.	+	.	.	.	30	+
1	10,000	.	.	.	+	.	30	+
10,000	100	+	.	.	.	.	36	+
100	100	+	.	.	.	.	36	+
1	100	.	.	+	.	.	36	+
100	1	+	.	.	.	.	39	+
1	1	.	+	.	.	.	36	+
32C.15	BC127							
10,000	10,000	.	.	+	.	.	30	+
100	10,000	.	.	+	.	.	30	+
1	10,000	.	.	.	+	.	27	+
10,000	100	+	.	.	.	.	36	±
100	100	.	+	.	.	.	33	±
1	100	.	.	+	.	.	30	+
100	1	.	+	.	.	.	45	±
1	1	.	+	.	.	.	45	±
32C.15	484							
10,000	10,000	+	.	.	.	.	30	±
100	10,000	.	.	+	.	.	33	±
1	10,000	.	.	.	.	+	36	+
10,000	100	+	.	.	.	.	30	±
100	100	.	.	+	.	.	36	+
1	100	.	.	+	.	.	36	+
100	1	+	.	.	.	.	36	±
1	1	.	+	.	.	.	39	+

Broken cream: − no broken cream observed. ± broken cream doubtful. + broken cream observed.

Table 2

Cultures	Approx. initial inoculum/ml. *Streptococcus* : Spore-former	First storage temperature	Actual colony count/ml. after 24 hr. storage		Ratio of spore-former : *Streptococcus*						Broken cream		
					After storage for 48 hr. at			At time of the end-point of the alcohol precipitation test					
			Streptococcus	Spore-former	15° C.	18° C.	22° C.	15° C.	18° C.	22° C.	15° C.	18° C.	22° C.
35 C.9 and BC60	1 : 100	5° C.	1	15	1 : 20	1 : 8	1 : 100	1 : >1,000	1 : 90	1 : 13	−	+	+
		10° C.	3	27	1 : 1	1 : 2	1 : 25	1 : >1,000	1 : 300	1 : 400	−	+	+
		15° C.	560	760	1 : 18	1 : 65	1 : 14	1 : 60	1 : 60	.	+	+	+
35 C.9 and BC127	1 : 100	5° C.	2	2	1 : 1	1 : 5	1 : 1	1 : 5	1 : 10	1 : 15	−	−	+
		10° C.	12	81	1 : 2	1 : 6	1 : 30	1 : 7	1 : 10	1 : 10	±	+	+
		15° C.	5,400	8,960	1 : 100	1 :50	1 : 100	1 : 30	.	.	+	+	+
35 C.9 and 484	1 : 100	5° C.	0	20	1 : >10	1 : 15	1 : 3	1 : >1,000	1 : 70	1 : 9	−	+	+
		10° C.	4	55	1 : 2	1 : 0·15	1 : 2·5	1 : 2	1 : 2·5	1 : 70	−	+	+
		15° C.	190	390	1 : 1	1 : 20	1 : 60	1 : 25	1 : 11	.	−	+	+
32 C.15 and BC60	1 : 100	5° C.	1	4	.	1 : 3	1 : 0·33	1 : 2	1 : 300	1 : 150	±	+	±
		10° C.	3	30	1 : 12	1 : 100	1 : 60	1 : >1,000	1 : 25	1 : 150	+	+	+
		15° C.	60	1,270	1 : 200	1 : 200	1 : 50	1 : 1	1 : 15	1 : 40	+	+	+
32 C.15 and BC127	1 : 100	5° C.	2	31	1 : >10	1 : 0·5	1 : 2	1 : >1,000	1 : 2	1 : 0·5	+	+	+
		10° C.	3	132	1 : 0·5	1 : 2	1 : 6	1 : >1,000	1 : 300	1 : 300	+	±	+
		15° C.	6,320	35,000	1 : >1,000	1 : 9	1 : 25	.	.	.	+	+	+
32 C.15 and 484	1 : 100	5° C.	7	9	.	1 : 30	1 : 0·25	1 : >1,000	1 : >1,000	1 : >1,000	−	+	+
		10° C.	0	123	1 : 9	1 : 0·3	1 : 0·17	1 : 150	1 : 6	1 : 25	−	+	+
		15° C.	46	760	1 : >1,000	1 : >1,000	1 : >1,000	1 : 8	.	1 : 170	−	+	+
12 and BC60	1 : 100	5° C.	163	3	1 : >500	.	1 : >1,000	1 : >1,000	.	1 : >1,000	−	.	±
		10° C.	147	1	1 : >1,000	.	1 : >1,000	1 : >1,000	.	1 : >1,000	±	.	+
		15° C.	15,000	45	1 : >1,000	.	1 : >1,000	1 : >1,000	.	.	±	.	+

Broken cream: − no broken cream observed. ± broken cream doubtful. + broken cream observed.

In ten of the fifty-four subsamples no broken cream was observed, and four of these subsamples showed no development of *B. cereus* as judged by the colony counts made after 48 hr. and at the end-point of the 68% alcohol precipitation test. It is of interest that four of the six subsamples stored at 5° C. for 24 hr. before being transferred to 15° C. failed to show broken cream. It has already been shown (6) that pure cultures of *B. cereus* in milk tended to die off when held at low temperatures, and it is suggested that the absence of broken cream in the subsamples was due entirely to the temperature of storage, the development of *Str. lactis*, which is fairly rapid at 15° C., being responsible for the ultimate spoilage of the milk. From the results obtained from those subsamples stored at higher temperatures it is obvious that a low proportion of lactic acid organisms in milk does not suppress the growth of *B. cereus* when the temperature is favourable. It is suggested that the observations of Rowlands & Provan on the predominance of *Str. lactis* at C.O.B. end-point of milk stored at 15° C. may have been due to the selective effect of that temperature.

No explanation can be offered for the fact that *B. cereus* was present in large numbers in the five remaining subsamples in which broken cream was not recorded. Although the spore-former developed sufficiently to produce broken cream in forty cases, it was outnumbered in every case by the *Streptococcus* at the end-point of the alcohol precipitation test and in twenty-three of the subsamples the final proportions of *Streptococcus* to spore-former were at least 50:1.

Our results confirm those obtained with mixtures of the two organisms in milk at 22° C., and it is concluded that, although the lactic streptococci are capable of more rapid growth than *B. cereus* and may ultimately cause spoilage of the milk, they do not suppress growth of *B. cereus* or the production of broken cream at temperatures favourable to the development of this organism. Finally, it is confirmed that an initial period of storage at 5° C. followed by 15° C. is unfavourable to the growth of *B. cereus* in milk.

The effect of a nisin-producing strain of Streptococcus lactis *upon the growth of* Bacillus cereus *in milk*

One strain of the spore-former (BC60) was grown in association with a nisin producing strain of lactic *Streptococcus*, no. 12 of Hirsch & Grinsted (11). The two organisms were transferred four times through litmus milk before inoculation. The subsamples stored at 18° C. were omitted, and it was found that only in those subsamples held at 22° C. after preliminary storage at 10 and 15° C. did broken cream develop. Comparing the results with those previously obtained, it is clear that the nisin-producing organisms did have some inhibitory effect at temperatures not entirely favourable for the development of *B. cereus*. However, at those temperatures where *B. cereus* was able to multiply rapidly, broken cream was recorded after 48 hr. storage. It may be noted that the colony count of *B. cereus* was found to decrease after 48 hr. up to the end-point of the alcohol precipitation test, presumably because of the effect of the antibiotic.

The associative growth of Bacillus cereus *and* coli-aerogenes *organisms in milk*

The effect of temperature upon the growth of *B. cereus* (BC60) in association with two strains of *coli-aerogenes* organisms isolated from pasteurized milk in which broken cream developed was studied. It was again found that the presence of the acid-producing

organisms did not affect the growth of *B. cereus* except at those temperatures which were known to be unfavourable to the growth of the pure culture.

CONCLUSION

It is concluded that unless the initial ratio of acid-producing bacteria to *B. cereus* is very large, as it may be in raw milk but not in pasteurized milk, broken cream is liable to develop, although the fault may be controlled under certain conditions of temperature whatever type of flora is present initially.

SUMMARY

It has been found that when three strains of *Bacillus cereus* are grown in turn with each of two strains of *Streptococci lactis* and two strains of the *coli-aerogenes* group, the growth of the spore-formers is neither suppressed nor stimulated. It has been confirmed, however, that the spore-former is unlikely to develop if the milk is held at 15° C. or below. Broken cream results if marked growth of *B. cereus* occurs. A nisin-producing strain of *Str. lactis* did not retard the development of the spore-former in milk, but after initial increase the numbers of *B. cereus* decreased. Broken cream was not prevented.

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REFERENCES

(1) STONE, M. J. & ROWLANDS, A. (1952). *J. Dairy Res.* **19**, 51.
(2) SORIANO, A. M. DE (1946). *Rev. Asoc. argent. Diet.* **4**, 63.
(3) ROWLANDS, A. & PROVAN, A. L. (1941). *Proc. Soc. agric. Bact.* p. 29.
(4) EDGELL, J. W. & BIRD, E. R. (1950). *J. Soc. Dairy Tech.* **3**, 171.
(5) GARVIE, E. I. & ROWLANDS, A. (1952). *J. Dairy Res.* **19**, 133.
(6) STONE, M. J. (1952). *J. Dairy Res*, **19**, 51.
(7) DAVIS, J. G. (1940). *Milk Tr. Gaz.* **10**, 4.
(8) GARVIE, E. I. & ROWLANDS, A. (1952). *J. Dairy Res.* **19**, 263.
(9) MILES, A. A. & MISRA, S. S. (1938). *J. Hyg., Camb.*, **35**, 732.
(10) ASHTON, T. R. (1950). *J. Dairy Res.* **17**, 261.
(11) HIRSCH, A. & GRINSTED, E. (1951). *J. Dairy Res.* **18**, 198.

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497. AN IMPROVED MEDIUM FOR LACTOBACILLI

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The classification of organisms of the genus *Lactobacillus* has been complicated by the failure of certain strains to grow consistently in the test media used. As a preliminary to a study of the physiological and biochemical characteristics of the lactobacilli, it was essential to find a medium in which all members of the genus would grow satisfactorily so that valid comparisons of the reactions of various strains could be made. Yeast dextrose litmus milk (1) supported growth of all the strains of lactobacilli in our collection, but a broth medium, which could be adapted for use in physiological tests, was required.

EXAMINATION OF MEDIA DESCRIBED IN THE LITERATURE

Kulp (2), Weiss & Rettger (3) and Valley & Herter (4) described tomato-juice media, and Nymon & Gortnor (5) gave details of a liver tryptone broth. Although some organisms were found to grow well in these media, the growth of certain strains which multiply rapidly in milk was poor. This suggested that a casein digest substrate might be suitable, but Orla-Jensen's (6) peptic digest medium, Roberts & Snell's (7) pancreatic digest medium, which is used in microbiological assays, the double digest medium described by Davis (8), a milk medium reported by Brown & Howe (9) and Hunter's (10) rennet digest medium were tested and found unsatisfactory for strains of *Lactobacillus bulgaricus* and *L. acidophilus*. No marked improvement followed the inclusion of various inorganic salts or a combination of casein digest and tomato juice. However, earlier observations in this laboratory had shown that the addition of a low concentration of bile salt improved the growth of some strains of *L. acidophilus*. Accordingly, the effects of other surface-tension depressants and fatty acids were investigated.

THE EFFECT OF THE ADDITION OF SURFACE TENSION DEPRESSANTS AND OTHER SUBSTANCES

Literature

Kodicek & Worden (11,12) observed an inhibitory effect of oleic acid on *L. helveticus*, but Sherman & Albus (13) found the presence of butterfat to be of value for other lactobacilli, and Hunter (14) reported that the beneficial effect of sodium oleate in a casein digest medium was greater than that of sodium ricinoleate or sodium glycocholate. More recently, Hutchings & Boggiano (15), Whitehill, Oleson & SubbaRow (16) and Tomarelli, Norris, Gyorgy, Hassinen & Bernhart (17) showed that oleic and other unsaturated fatty acids stimulated the growth of various strains of lactobacilli. Williams & Fieger (18) described the stimulatory effects on lactobacilli of non-ionic detergents, particularly those containing oleate, and Williams, Broquist & Snell (19), Kitay & Snell (20) and Rogosa & Mitchell (21) observed the beneficial effects of various Tween compounds. Davis & Dubos (22) developed a medium for *Mycobacterium tuberculosis* in which bovine serum

albumin was added to a medium containing Tween in order to bind traces of free oleic acid, and Williams & Fieger (23) observed a reduced lag phase when *L. casei* was grown in an oleic acid medium in which albumin was included. Ley & Mueller (24) reported that an inhibitory effect of agar on *Neisseria gonorrhoea*, due to fatty acids, was neutralized by starch, and this suggested that albumin, which cannot be heat-sterilized, might be replaced by starch. It appeared that an examination of the effects of certain fatty acids, surface tension depressants and of albumin and starch might prove profitable.

Experimental observations

Preliminary experiments showed that, although bile salt and sodium tauroglycocholate had some growth-promoting effect, either Tween 80 or sodium oleate in tomato juice or casein digest media was preferable and gave remarkable increases in the growth of many species of lactobacilli. With the further addition of albumin or starch, still better results were obtained. Subsequently, tomato dextrose broth, a modified form of Valley & Herter's (4) medium, was found to be more suitable than casein digest as a basic medium. Tween 80 had a slightly greater stimulatory effect than Tween 20 or Tween 85, and there were indications that sodium oleate was less satisfactory than Tween 80, as it appeared to produce a longer lag phase for many strains, particularly *L. bulgaricus*. In addition, some difficulty was found in preparing a clear medium containing oleate. Yeastrel was also found to increase growth in these media.

The evidence so far obtained did not justify definite conclusions, as small differences were recorded between the growths of a given organism in the different media tested, and variable results were obtained when different strains were grown in a single medium. However, the experiments referred to previously had shown that the greatest effects were obtained in a tomato juice basal medium with Tween 80, sodium oleate, albumin, starch and Yeastrel. Since the object of this work was to obtain a single medium in which all strains would grow satisfactorily, the optimum concentrations and combinations of these substances were determined by means of statistically planned experiments.

Statistical experiments

Test organisms

Species which had proved the most difficult to grow in the preliminary experiments were chosen for this work. Six strains of *L. acidophilus*, four of *L. bulgaricus*, two of *L. delbrückii*, one of *L. pastorianus* and six strains of recently isolated unidentified intestinal lactobacilli were used.

Media

The basal medium used throughout this work was tomato dextrose broth of the following composition:

	%
Tomato juice	40·0
Neopeptone	1·5
Dextrose	2·0
NaCl	0·5
Distilled water	to 100·0

pH 6·8

The tomato juice was prepared by adding 1 lb. of tomatoes to a litre of distilled water, steaming until pulped (about 1½ hr.), filtering and autoclaving at 15 lb. for 20 mins. The

juice was adjusted to pH 7·0 before use. Tween 80, sodium oleate, bovine serum albumin fraction V, soluble starch and Yeastrel were added to the basal broth in varying concentrations and combinations. All media were autoclaved, Seitz-filtered albumin solution being added aseptically where necessary.

Methods

A standard inoculum from a 48 hr. culture of the organism growing in the basal tomato dextrose broth was used. All media were inoculated in triplicate with each organism, and the order of inoculation was randomized. Optical densities were read in randomized order after 24, 48 and 96 hr. incubation.

Three consecutive experiments were made, the results of one being used to plan the next.

Results

The results of the first experiment confirmed earlier impressions that all the additional substances under test caused increases in growth. In the second experiment it was found that a medium containing Tween 80 and albumin was slightly better than other media. The practical difficulties of preparing such a medium for routine use, however, were considered to outweigh its slight superiority, and the testing of media containing albumin was discontinued.

The results of the third and final experiment showed that two media were generally superior to all others, and these differed only in that one contained starch. Before this particular experiment was complete the starch-containing medium had been used successfully in preliminary work on the classification of the lactobacilli. This has been chosen, therefore, as the standard medium in that investigation, and has the following composition:

	%
Tomato juice	40·0
Neopeptone	1·5
Dextrose	2·0
NaCl	0·5
Tween 80 (polyoxyethylene sorbitan mono-oleate)	0·1
Yeastrel	0·6
Soluble starch	0·05
Distilled water	to 100·0
pH 6·8	

The addition of agar to the standard medium provides a good solid medium for lactobacilli.

The experimental evidence and statistical results of this work will be published in detail later.

DISCUSSION

Although the standard medium contained a variety of substances which have been shown to stimulate the growth of these organisms, Tween 80 (polyoxyethylene sorbitan mono-oleate) appeared to be of paramount importance. The precise mechanism of its action is uncertain, but, since sodium oleate has a similar, though smaller effect, it is possible that oleate is utilized by the bacterial cells in their metabolism (Hunter (14) and Dubos (25)). Experience with this medium has shown that, not only does it support excellent growth of recalcitrant strains such as *L. acidophilus* and *L. bulgaricus* which were used as test

organisms, but that the growth of varieties with less exacting requirements, such as *L. casei* and *L. plantarum*, is also considerably improved. It is of interest that freshly isolated strains of *L. pastorianus*, inoculated directly from beer, grow well in this medium. It may be that the inclusion of soluble starch in the medium is of little value, but nevertheless it is possible that starch may improve the growth of some species of lactobacilli which were not included in the critical tests. For this reason and also because the medium has proved to be adequate for the purpose for which it was developed, we have continued to include starch in the standard medium. Although the medium containing albumin was rejected as impracticable, it is perhaps worth further consideration. Modification of the standard medium to contain 0·05% albumin in place of the soluble starch might be an advantage in special work.

All the organisms in our collection of lactobacilli, comprising nearly 450 strains which include representatives of all recognized species, grow well, consistently and give reproducible results in the standard medium. The adoption of this medium as the basis of certain biochemical and physiological tests has made possible a classification of the lactobacilli, the details of which will be published later.

SUMMARY

Several media previously recommended for lactobacilli by other workers have been examined, but none was found to support good growth of all species. Experiments are described in which the optimum concentrations and combinations of various ingredients, particularly Tween 80, were determined. These resulted in the development of a practical medium which has proved satisfactory for the growth of all varieties of lactobacilli.

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REFERENCES

(1) DAVIS, J. G. (1935). *J. Dairy Res.* **6**, 121.
(2) KULP, W. L. (1927). *Science*, **66**, 512.
(3) WEISS, J. E. & RETTGER, L. F. (1933). *J. Bact.* **28**, 501.
(4) VALLEY, G. & HERTER, R. C. (1935). *Science*, **82**, 14.
(5) NYMON, M. C. & GORTNOR, W. A. (1946). *J. biol. Chem.* **163**, 277.
(6) ORLA-JENSEN, S. (1942). *The Lactic Acid Bacteria*, 2nd ed. Copenhagen: Ejnar Munksgaard.
(7) ROBERTS, E. C. & SNELL, E. E. (1946). *J. biol. Chem.* **163**, 499.
(8) DAVIS, J. G. (1936). *Proc. Soc. agric. Bact.*, Notes on the preparation of media.
(9) BROWN, J. H. & HOWE, P. E. (1922). *J. Bact.* **7**, 511.
(10) HUNTER, G. J. E. (1950). *J. Dairy Res.* **17**, 79.
(11) KODICEK, E. & WORDEN, A. N. (1945). *Biochem. J.* **39**, 78.
(12) KODICEK, E. & WORDEN, A. N. (1946). *Nature, Lond.*, **157**, 587.
(13) SHERMAN, J. M. & ALBUS, W. R. (1922). *Abst. Bact.* **6**, 17.
(14) HUNTER, C. A. (1924). *Abst. Bact.* **8**, 3.
(15) HUTCHINGS, B. L. & BOGGIANO, E. (1947). *J. biol. Chem.* **169**, 229.
(16) WHITEHILL, A. R., OLESON, J. J. & SUBBAROW, Y. (1947). *Arch. Biochem.* **15**, 31.
(17) TOMARELLI, R. M., NORRIS, R. F., GYORGY, P., HASSINEN, J. B. & BERNHART, F. W. (1949). *J. biol. Chem.* **181**, 879.
(18) WILLIAMS, V. R. & FIEGER, E. A. (1947). *J. biol. Chem.* **170**, 399.

(19) Williams, W. L., Broquist, H. P. & Snell, E. E. (1947). *J. biol. Chem.* **170**, 619.
(20) Kitay, E. & Snell, E. E. (1950). *J. Bact.* **60**, 49.
(21) Rogosa, M. & Mitchell, J. A. (1950). *J. Bact.* **59**, 303.
(22) Davis, B. D. & Dubos, R. J. (1947). *J. exp. Med.* **86**, 215.
(23) Williams, V. R. & Fieger, E. A. (1949). *J. biol. Chem.* **177**, 739.
(24) Ley, H. L. & Mueller, J. H. (1946). *J. Bact.* **52**, 453.
(25) Dubos, R. J. (1947). *J. exp. Med.* **85**, 9.

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498. SOME GROUP N STREPTOCOCCI ISOLATED FROM RAW MILK

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A number of strains of streptococci falling into serological group N which could not be identified as either *Streptococcus lactis* or *Str. cremoris* were isolated during a study of the bacterial flora of raw milk (1). These organisms were dominant in milk incubated at 22° C. until the end-point of the clot-on-boiling test in three out of the twelve samples examined and, apparently, had played a major part in their spoilage. Similar strains were isolated in smaller numbers from the samples aged under other temperature conditions.

These strains of group N were of considerable importance in the milks examined, but reports of similar organisms do not appear in the literature.

MATERIALS AND METHODS

Isolation of cultures

Samples from twelve local farms were taken immediately after the evening milking and stored at 14° C. overnight. Between 9 and 10 a.m. on the following day (initial) the samples were examined by the methylene-blue reduction test at 37·5° C. and for keeping quality, as measured by the clot-on-boiling test (1) at 22° C. Suitable dilutions were plated in yeastrel milk agar, initially, at the time of reduction of the methylene-blue test and at the time of the end-point of the clot-on-boiling test. The initial plates were incubated at 37·5° C. for 3 days and at 22° C. for 5 days; those prepared at the time of dye reduction were incubated at 37·5° C. for 3 days, and those prepared at the end-point of the clot-on-boiling test at 22° C. for 5 days. Approximately twenty-five colonies were picked from a suitable plate of each set and inoculated into litmus milk. After purification on yeastrel milk agar, the cultures were examined physiologically and serologically.

Physiological tests

Growth at 10° C. was observed in yeast dextrose broth after 7 days' incubation at 10 ± 1° C.

Growth at 40° C. was observed in litmus milk after 48 hr. incubation at $40 \pm 0{\cdot}1$° C. (2)

Survival at 60° C. *for* 30 *min.* The method of Shattock (3) was used.

Growth at pH 9·6 *and* 9·2. The method of Shattock & Hirsch (4) was followed. For tests at pH 9·2 the broth was adjusted to pH 9·4 before overnight storage in the ice chest.

Growth in sodium chloride broth. The required amount of sodium chloride was added to the basal medium described by Swartling (5). Tests were made at 30° C. and the results observed after 3 days.

Arginine hydrolysis. The method of Abd-el-Malek & Gibson (2) was followed.

Growth in the presence of methylene blue. Cultures were grown in skim milk containing 1:20,000 parts of methylene blue. Reduction or partial reduction of the dye after 5 days' incubation at 30° C. was recorded as positive.

Production of gas from glucose. The method of Gibson & Abd-el-Malek (6) was used with the medium containing tomato juice.

Haemolysis. The method of Brown (7) modified by Shattock (3) was used.

'Carbohydrate' fermentation was observed in peptone water containing 0·5% (w/v) of the carbohydrate (0·1% for inulin and aesculin).

Serological tests

Precipitin tests. HCl extracts (8) were tested by the precipitin test against specific sera for groups B, C, D, E and N, using the methods described by Shattock (3). The extracts were concentrated (fraction A Shattock (3)) and retested against the sera for groups D and N.

RESULTS

The eighty-one strains were isolated from seven samples and fifty-six were from milk at the time of the end-point of the clot-on-boiling test. Only thirteen strains were isolated from plates incubated at 37·5° C., and of these ten were from one sample (25) at the time of the reduction of methylene blue, the remaining three cultures were from the initial milk of sample 31. These results suggest that a temperature of 37·5° C. is probably unfavourable for the organisms.

The physiological and serological reactions of the cultures are given in Table 1. Results are included from strains which were classified serologically and from strains apparently related but not grouped. When either the crude or concentrated extracts were examined, reactions were observed only with sera of group N.

The cultures do not form an entirely homogeneous collection, but they have many characteristics in common. They fail to grow at pH 9·2 or in the presence of 4% NaCl, to hydrolyse arginine, to produce gas from glucose and to ferment sorbitol or inulin. The majority grow at 10° C., in the presence of methylene blue and in the presence of 2% NaCl, and several reduce litmus rapidly and ferment mannitol, raffinose, sucrose, aesculin, maltose and dextrin.

DISCUSSION

The limits of variation within the species of *Str. lactis* and *Str. cremoris* are reasonably well defined in the literature. *Str. lactis* grows at pH 9·2 in 4% NaCl and at 40° C. (9); it produces ammonia from arginine (9) and ferments maltose and dextrin (10). With these same tests, *Str. cremoris* gives negative results. *Str. lactis* ferments a wider range of carbohydrates than *Str. cremoris* (11), and strains of *Str. lactis* fermenting mannitol, sucrose and raffinose have been reported (12,13). The organisms therefore possess characteristics of both *Str. lactis* and *Str. cremoris* combined. Serologically these organisms fall into group N, yet biochemically the reactions are suggestive of some strains of group D, in particular *Str. bovis*, which ferments sucrose, raffinose, maltose and dextrin, but not mannitol (3, 11). Thirteen strains ferment mannitol, fourteen raffinose and all twenty-seven ferment sucrose; only three fail to ferment maltose and only two dextrin.

In litmus milk only the strains from samples 23, 25 and some from sample 30 give reactions similar to *Str. lactis* or *Str. cremoris*, i.e. a rapid reduction of the litmus, followed by acidity and curdling of the milk; but on first isolation many of the strains, particularly those from samples 30 and 31, were difficult to culture in milk. Collins, Nelson & Parmelee (14) report that heated milk is deficient in nutrient requirements for *Str. cremoris*,

Table 1. *Physiological and serological reactions*

Table 1. *Physiological and serological reactions*

No. of cultures examined	No. of cultures represented	Sample no.	Source of culture	Growth at 10° C.	Growth at 40° C.	Growth at pH 9·2	Growth in 4% NaCl	Growth in 2% NaCl	Growth in methylene-blue milk	Survival at 60° C. for 30 min.	Hydrolysis of arginine	Gas produced from glucose	Litmus milk reaction	Haemolysis	Fermentation tests: Mannitol	Raffinose	Sucrose	Aesculin	Maltose	Dextrin	Sorbitol	Inulin	Serological group
1	10	23	C	+	−	−	−	+	+	−	−	−	RAC	Sl. green	−	+	+	+	+	+	−	−	N
1	10	25	D	−	−	−	−	±	+	−	−	−	RAC	−	−	−	+	+	+	+	−	−	N
1	4			+	−	−	−	+	+	−	−	−	RA	Sl. green	+	+	+	+	+	+	−	−	N
1				+	−	−	−	+	±	−	−	−	RAC	Sl. green	+	+	+	+	+	+	−	−	N
1	9	30	C	+	−	−	−	+	±	−	−	−	RAC	−	−	−	+	+	+	+	−	−	N
1				+	−	−	−	+	±	−	−	−	−	Sl. green	−	−	+	+	+	+	−	−	N
1	1			+	−	−	−	+	+	−	−	−	A	Sl. green	−	+	+	+	+	+	−	−	N
1	1			+	−	−	−	+	+	−	−	−	ACr	Green	+	+	+	+	+	+	−	−	N
1	2	31	37	+	−	−	−	+	+	−	−	−	−	−	+	+	+	+	+	+	−	−	N
1	1	31	C	+	−	−	−	+	+	−	−	−	−	Sl. green	+	+	+	+	+	+	−	−	I
0	1	31	37	+	−	−	−	+	−	−	−	−	RA	Sl. green	−	−	+	+	+	+	−	−	N
1	3	31	22	+	−	−	−	−	−	−	−	−	RA	−	−	−	+	+	+	+	−	−	N
1	4	31	C	+	−	−	−	−	±	−	−	−	A	−	−	−	+	+	+	+	−	−	N
1	1	31	22	+	−	−	−	+	+	−	−	−	A	Sl. green	+	+	+	+	+	+	−	−	N
1	1	31	C	+	−	−	−	+	+	−	−	−	Ar	Sl. green	+	+	+	+	+	+	−	−	I
1	3	32	22	+	−	−	−	+	+	−	−	−	Ar	−	−	−	+	+	+	+	−	−	N
1	3	32	C	+	−	−	−	+	+	−	−	−	Ar	Green	+	+	+	+	+	+	−	−	N
1	2	33	22	+	−	−	−	±	+	−	−	−	Ar	Sl. green	2+ 2−	+	+	+	+	+	−	−	N
4	16	33	C	+	−	−	−	±	±	−	−	−	Ar	Sl. green	+	−	+	+	+	+	−	−	I
0	1	34	22	−	−	−	−	+	+	−	−	−	A	Sl. green	+	−	+	+	+	+	−	−	I
1	3	34	C	−	−	−	−	+	+	−	−	−	a	−	−	−	+	−	−	−	−	−	I
1	1	34	C	−	−	−	−	−	−	−	−	−	Ra	−	−	−	+	−	−	−	−	−	I

Litmus milk reactions:
R = complete reduction.
A = acid.
C = clot.
r = slight reduction.
a = slight acid.

Source of culture:
C = isolated at time of end-point of C.O.B.
D = isolated at time of dye reduction.
37 = isolated from initial milk plates held at 37·5° C.
22 = isolated from initial milk plates held at 22° C.

and the same explanation might apply to our cultures. Support for this suggestion was gained during the course of another investigation, when it was observed that, if only 100,000 cells of some of the strains were inoculated per ml. of aseptically drawn pasteurized milk (63° C. for 30 min.) and subsequently incubated at 22° C., they failed to grow. However, if the inoculum was increased to 1,000,000 cells per ml. the milk rapidly developed a high acidity. Under the same conditions only 1 cell of *Str. lactis* per ml. would grow and cause spoilage of the milk.

The temperature at which a sample of milk is held prior to plating out and the length of the incubation period will help in selecting the organisms isolated. While it has been claimed that *Str. cremoris* is an adaptive species not found in raw milk (14), it has been isolated from this source under certain conditions (2), and Nichols & Hoyle (12) ascribe their failure to isolate *Str. cremoris* directly to the method of treatment of the milk. It is probable that the treatment of the milk selected during the present investigation, particularly incubating the samples at 22° C., is suited for the development of group N streptococci and may explain why strains similar to those now described have not been reported elsewhere.

Because of the still scanty knowledge of the strains in group N it is not possible to suggest how these cultures are related to other strains within the group.

REFERENCES

(1) Garvie, E. I. & Rowlands, A. (1952). *J. Dairy Res.* **19**, 133.
(2) Abd-el-Malek, Y. & Gibson, T. (1948). *J. Dairy Res.* **15**, 233.
(3) Shattock, P. M. F. (1949). *J. gen. Microbiol.* **3**, 80.
(4) Shattock, P. M. F. & Hirsch, A. (1947). *J. Path. Bact.* **59**, 495.
(5) Swartling, P. F. (1951). *J. Dairy Res.* **18**, 256.
(6) Gibson, T. & Abd-el-Malek, Y. (1945). *J. Dairy Res.* **14**, 35.
(7) Brown, J. H. (1919). *Mem. Rockefeller Inst. Med. Res.* **9**.
(8) Lancefield, R. C. (1933). *J. exp. Med.* **57**, 571.
(9) Yawger, E. S. & Sherman, J. M. (1937). *J. Dairy Sci.* **20**, 205.
(10) Hunter, G. J. E. (1946). *J. Hyg., Camb.*, **44**, 264.
(11) Orla-Jensen, S. (1943). *The Lactic Acid Bacteria* (Erganzungsband). Copenhagen: Ejnar Munksgaard.
(12) Nichols, A. A. & Hoyle, M. (1948). *J. Dairy Res.* **15**, 409.
(13) Orla-Jensen, A. & Hansen, P. A. (1932). *Zbl. Bakt.* II. Abt. **86**, 6.
(14) Collins, E. B., Nelson, F. E. & Parmelee, C. E. (1950). *J. Bact.* **59**, 69.

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499. THE INHIBITION OF MICRO-ORGANISMS BY RAW MILK

I. THE OCCURRENCE OF INHIBITORY AND STIMULATORY PHENOMENA. METHODS OF ESTIMATION

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(With 7 Figures)

INTRODUCTION

It has been known for the last sixty years that milk, like serum, can inhibit the growth of micro-organisms, and much work has been done on this subject. The substances such as enzymes and antibodies, known for their antibacterial activity, which may occur in milk, have been shown not to be the main constituents of the inhibitory substances of milk *in vitro*. Jones (1, 2, 3, 4, 5) has contributed the most detailed work on the inhibition of streptococci by raw milk, and the substance he investigated was called 'lactenin'. Jones (1) has reviewed the literature up to 1927.

A subject of this nature is complex and may be approached from several angles. Thus Singh & Laxminarayama (6) studied the growth of 'starter' organisms in milk. They found that pasteurization and boiling improved the growth and activity of the starter in ordinary milk, especially with early and advanced lactation milks. They confirmed the findings of various authors (7, 8) that mastitis milk reduces the rate of growth of starter organisms when compared with normal milk. They thought that some antibacterial substances were responsible for this. However, Davis & McClemont (9) pointed out that the slow growth of starters in mastitis milk may be related also to its lower content in bacterial growth factors (10). This interaction of inhibitory substances and growth factors has been previously observed by Orla-Jensen (11, 12). Our own results show that the type of response obtained depends largely on the test organism and the medium used. The inhibition of the micro-organisms commonly responsible for milk spoilage may also be of interest with regard to the keeping quality of milk. This point has been discussed by Hobbs (13), while Morris & Edwards (14, 15) noted the occurrence in raw milk of a bacterial substance specific for certain coliform organisms.

One of the early observations of Jones was that *Streptococcus pyogenes* was more susceptible to the inhibitory action of raw milk than *Str. agalactiae*. Pullinger & Kemp (16) have followed up this observation. They found that, in milk stored at room temperature, *Str. pyogenes* died out relatively quickly, even if the initial count was heavy. *Str. pyogenes* milk-borne epidemics are not therefore likely to be due to the growth of the organisms in the milk during storage and handling, and the natural inhibitory properties of milk appear to play an important part in this. The effect of raw milk on other micro-organisms, and especially the causative organisms of cholera, typhoid and diphtheria, has been studied by earlier workers (17, 18), but it is well known that raw milk can be dangerous (see, for instance, (19)). Wilson & Rosenblum (22) failed to protect mice, which were infected with *Str. pyogenes* by feeding raw milk.

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The present investigation arose from the epidemiology of bovine mastitis. Jones (1) was responsible for the idea that cows resistant to mastitis produced milk with a higher inhibitory titre than susceptible cows, but his figures are not, in themselves, fully convincing. Later he was able to show that *Str. agalactiae* became adapted to raw milk and that, after a delay of some 6 hr., his cultures grew normally (4). Folley & Lee (23) used this observation to explain the time lag followed by the sudden appearance of large numbers of *Str. agalactiae* in the udder. More recently McEwen & White produced an artificial mastitis by infusing cultures of *Str. agalactiae*, but the inhibitory titre of the samples remained unaltered (24). They confirmed Jones's observation that colostrum is less active than milk, and Neave, Dodd & Henriques (25) have suggested that the udder is more susceptible to infection during the dry period than during lactation.

The apparent correlation between resistance to infection and the inhibitory power of raw milk, is however, rather uncertain in view of the results of Wilson & Rosenblum (20, 21, 22). They have shown that raw milk is only inhibitory under aerobic conditions, and that anaerobic conditions and reducing agents like cysteine and glutathione abolish the inhibitory action. When milk was removed from the udder by catheter, it decolorized methylene blue rapidly. Because of the reducing conditions prevailing in the udder, Wilson & Rosenblum suggested that there was no inhibitory action *in vivo*.

On the whole, our results confirm the findings of Wilson & Rosenblum. Our method of measuring the effect of raw milk on streptococci is, however, different from the methods previously employed. The measurement of the overall activity was a simple dilution assay similar to that used by other workers, but we set out with the *a priori* conception of a very complex phenomenon, the total effect being due to the interaction of different groups of substances. The simplest way of separating these effects was to use a quantitative dose-response curve, and to use the shape of the curve obtained as a pointer to further experimentation.

We have obtained by our assay method strong indications (and confirmatory experiments are described) that at least two substances are responsible for the inhibitory power of milk and that stimulatory substances, interfering with this inhibition, also occur.

EXPERIMENTAL

Diffusion cup assays

Technique. This well-known technique has been described in detail by Heatley (26). Some slowly diffusing antibacterial substances may not show up in this test and therefore in some of our experiments, prior to incubation, half the plates for each assay were refrigerated overnight to allow the diffusion of such substances in the agar before the growth of the test organism (27).

The possibility that the antibacterial action of milk was due to lysozyme has been discussed at length by Thompson (28). Therefore a preparation of crystallized lysozyme, obtained from Armour Laboratories Ltd., was included for comparison in some of the assays. Normal cow's milk and colostrum, and human milk rich in lysozyme (29) were tested using two test organisms: *Micrococcus lysodeikticus*, sensitive to lysozyme, and a strain of *Str. pyogenes* (Richards). The latter organism has already been shown by Jones to be very susceptible to inhibition by milk and much of the work described in this paper has been done with this organism.

Results. In diffusion assays *Str. pyogenes* was inhibited neither by milk nor by lysozyme, and overnight refrigeration did not affect these results.

Some of the results obtained using *M. lysodeikticus* as the test organism are given in Table 1. After 3 days' incubation, human milk and lysozyme gave large clear zones of inhibition surrounded by a halo of lysed colonies, whereas cow's milk and colostrum had no such effect. When the effect of the weight of the inoculum was tested (Table 2) it was found that, as the inoculum was decreased, so the effect of lysozyme became more uncertain, smaller zones usually being obtained with a decreasing inoculum. With milk, however, decreasing the inoculum resulted in clear, if small, zones of inhibition. With lysozyme, the clear zones became larger with increasing time of incubation, but with milk the zones became smaller and less well defined.

Table 1. *The assay of lysozyme by a diffusion technique*

	Dilution	Diameter of zones in mm. (av. of 4 replicates)		Dilution	Diameter of zones in mm. (av. of 4 replicates)
Crystalline lysozyme 0·1 % solution in saline	1/100	27	Cow's milk	Undiluted	10½
	1/200	22		1/2	9
	1/400	16½		1/4	–
	1/800	13½		1/8	–
Human milk	1/2	29	Cow's colostrum	Undiluted	9½
	1/4	25		1/2	–
	1/8	21½		1/4	–
	1/16	17		1/8	–

Plates inoculated with a suspension of *Micrococcus lysodeikticus* diluted 1/1000. Incubation for 3 days at 37° C. – =no zones.

Table 2. *The effect of the weight of inoculum of* Micrococcus lysodeikticus *on zones of inhibition and lysis with lysozyme and cow's milk*

		Dilutions of a suspension of *M. lysodeikticus*				
		1/100	1/1000	1/10,000	1/100,000	1/1,000,000
	Dilution	Diameter of zones in mm. (av. of 4 replicates)				
Crystalline lysozyme	1/100	25	25	27	24	16
0·1 % solution	1/200	22	22	21	20	13
Cow's milk	Undiluted	–	11	14½	16	15
	1/2	–	10	12	13	11½

Plates incubated for 3 days at 37° C. – = no zones.

The inhibitory power of cow's milk was further differentiated from lysozyme by dilution assays (see below). Lysozyme added to sterilized milk did not inhibit *Str. pyogenes*, whereas raw milk did (Table 3). Lysozyme retains its activity after being kept at 100° C. for 30 min. at pH 4·0 (28), while, as will be shown subsequently, the inhibitory substances of milk are most stable at pH 6·5 and, at this pH, are destroyed by heating for 20 min. at 71–74° C.

Discussion. With the samples tested, no zones of inhibition could be obtained by the diffusion cup method against *Str. pyogenes* and the zones obtained with cow's milk against *M. lysodeikticus* were too small and uncertain for a quantitative estimation. This method of assay was not used again. It is, however, simple and sensitive for lysozyme estimations. This diffusion method has, moreover, served to differentiate clearly between lysozyme and lactenin. From the results obtained, it can be concluded that the anti-streptococcal substance in milk is not lysozyme.

The behaviour of lysozyme is unusual in that it gives smaller zones as the weight of the inoculum of the test organism is decreased. There seems to be no previous record of this observation.

Table 3. *The differentiation of lactenin from lysozyme by dilution assays*

	Dilutions in autoclaved milk							
	1/2	1/4	1/8	1/16	1/32	1/64	1/128	0
Raw milk	−	−	−	−	−	−	+ + + +	+ + + +
Autoclaved milk +0·001% lysozyme	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +

Test organism: *Streptococcus pyogenes* 'Richards'. Incubation 20 hr. at 37° C. − = no growth of the test organism. + + + + = maximum growth of the test organism.

pH *response assays*

With streptococci, acid production is highly correlated with growth, and this property was chosen as the measure of growth response. pH measurements can be done rapidly and accurately and are not affected by the turbidity of the medium. The method also avoids errors due to non-specific agglutination (17,1) which make the interpretation of the results of a counting method difficult.

Technique. The test organism was *Str. pyogenes*, strain 'Richards', cultured in serum broth. Samples of milk were centrifuged to remove the fat and were then heated to 58° C. for 20 min. (1). The heating, which does not affect the inhibitory substances of milk (see below), was always sufficient, with the milk used, to reduce contamination to an insignificant level. Complement was also probably destroyed. A sample of milk from a normal cow in mid-lactation was chosen as a standard. After heat treatment, some of this was freeze-dried; other parts of the sample were stored in a refrigerator at −25° C. and at 1–2° C. The freeze-dried sample lost some of its activity, but the samples stored in the ordinary refrigerator remained stable for months. The standard was inhibitory to the test organism at 1/100 by the dilution method and was defined as containing 100 units of inhibitory substance (lactenin) per ml. of milk.

The procedure for assay was as follows: dilutions in powers of 1·5 or 2 were prepared in sterile separated milk adjusted to pH 7·0. The experiment was arranged so that a 1 ml. quantity of these dilutions was placed in each tube ($6 \times \frac{5}{8}$ in.), which also received 1 ml. of glucose-peptone-broth already inoculated with 5% v/v of an 18 hr. serum-broth culture of *Str. pyogenes*. The tubes were incubated at 37° C. for 6 hr., then 10 ml. of distilled water was added to each tube and the pH determined electrometrically.

Results. Example 1 represents the response obtained with the standard during a series of experiments (Fig. 1). This type of curve was frequently obtained. It suggests that a stimulatory and at least two inhibitory substances are involved.

Example 2 shows the responses obtained with a standard, whey (obtained by acid precipitation), dialyzed milk and dialysed whey (Fig. 2). With whey and the two dialysed preparations the stimulatory property is much more evident, probably because the low buffering power of these preparations permitted greater pH changes in the tubes containing a significant quantity of the sample.

Example 3 was chosen from a series of experiments in which colostrum and milk were assayed (Fig. 3). It is evident that the responses of colostrum and milk were qualitatively different.

Discussion. The complex nature of the curve, illustrated in Fig. 1, made this method difficult to use for estimations, and the straight-line responses, necessary for calculation, were not always obtained. A simpler dilution assay had to be used to measure

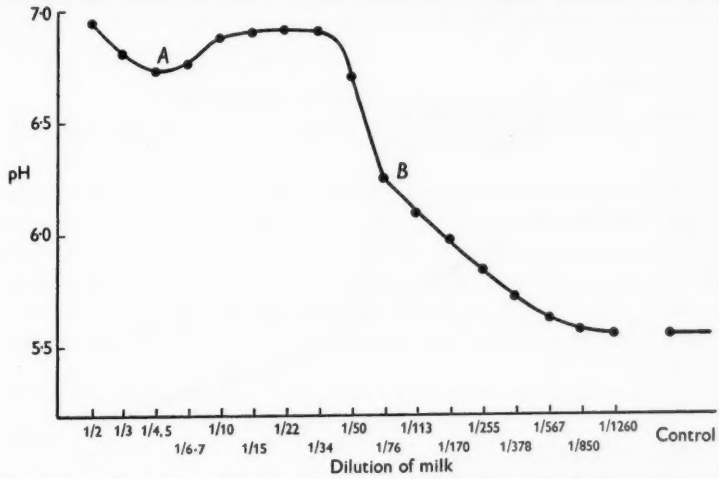

Fig. 1. The inhibitory effect of raw milk on the acid production of *Str. pyogenes*. Dose-response curve obtained with a sample of milk used for standard. Diluent = ½ milk, ½ glucose-peptone-broth. Incubation 6 hr. at 37° C.

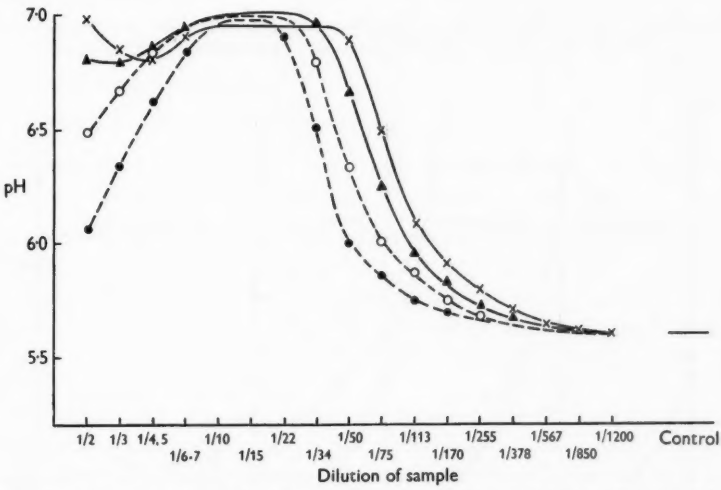

Fig. 2. Dose-response curves obtained with: × = milk, ▲ = whey, ○ = dialysed milk, ● = dialysed whey. Test organism = *Str. pyogenes*.

the total activity, but the results are not amenable to statistical analysis employed in quantitative assay work.

However, the dose-response curves were useful as a guide to the type and number of substances which were involved when measuring the effect of milk on *Str. pyogenes*. The first part of the curve (point *A* in Fig. 1) shows that the inhibition is at this point

somewhat diminished probably by a stimulatory substance which could be out-diluted by the inhibitory substance. The break in the response line at point *B* suggests that there are at least two inhibitory substances. The interaction of three substances may explain the shape of the curve in Fig. 1. The graph in Fig. 4 was prepared to illustrate hypo-

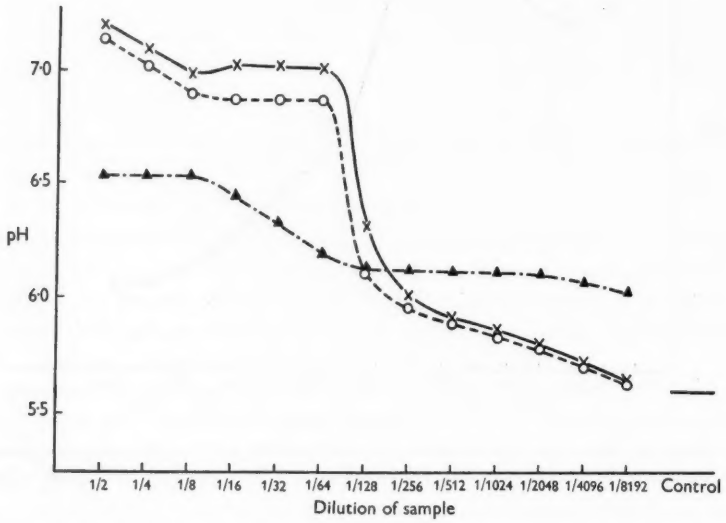

Fig. 3. Dose-response curves obtained with mid-lactation milk and colostrum. × = standard, ○ = Pandora (mid-lactation), ▲ = Rosalie 37 (colostrum). Test organism = *Str. pyogenes*.

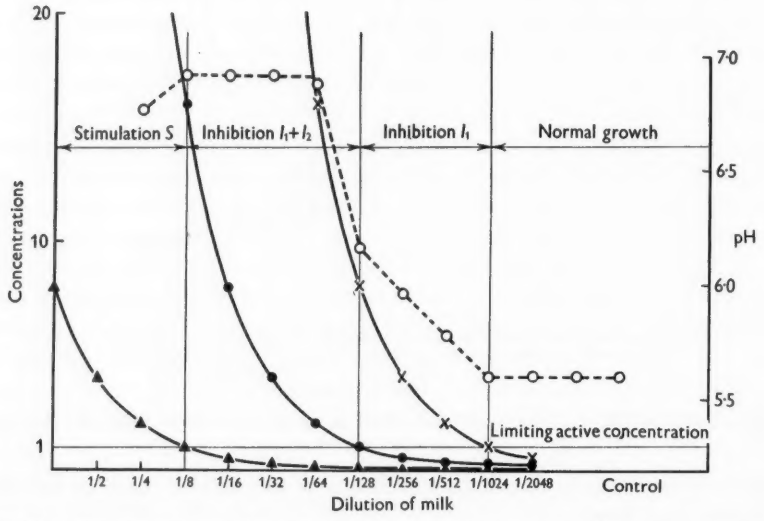

Fig. 4. Effect of decreasing concentrations of two inhibitory substances and one stimulatory substance on the acid production of *Str. pyogenes* (concentrations are in hypothetical units). ○ = dose-response of the test organism to raw milk, × = inhibitory substance I_1, ● = inhibitory substance I_2, ▲ = stimulatory substance S.

thetically how changes in the concentration of these substances, as the dilutions are made, might determine the type of response given by the test organism.

As with increasing dilutions the ratio of the substances is constant, it can be assumed that the stimulatory action is not due to a specific 'antagonizer' diminishing the action of the inhibitory substances, but that the stimulatory substance merely favours the growth of the test organism.

Estimations of the total inhibitory action

Technique. Samples, standard and culturing conditions were the same as in the previous method.

Dilutions of standard and unknown were made in sterile separated milk at pH 7·0 and distributed in one ml. quantities. Each tube then received one ml. of sterile milk at pH 7·0 containing (*a*) 0·5% peptone (Evans), (*b*) 0·004% bromocresol purple, (*c*) 0·05% of an 18 hr. culture of *Str. pyogenes* 'Richards'. The tubes were incubated at 37° C. for 20 hr. Because a standard was used with every assay, results could be expressed easily in terms of units, the titre of an assay being taken as the last tube to show no acid production.

Table 4. *The reproducibility of the dilution method for the estimation of the total inhibitory action of milk*

Samples		Replicates on three different days 1	2	3
Charis (mid-lactation)	(RF)	100	100	125
	(RH)	150	100	225
Valerie (mid-lactation)	(RH)	30	30	37
	(LH)	30	25	20
Winnie 11 (1st day after calving)	(RF)	335	280	280
	(LF)	335	280	280
Clara 15 (3rd day before calving)	(RF)	9	9	–
	(RH)	15	15	–
Clara 15 (1st day after calving)	(RF)	10	9	–
	(RH)	15	9	–
Bulk Milk, M_3		100	125	100

Figures are units of lactenin per ml. of sample. – =not done.

Results. Reproducibility of the test. Table 4 gives the results of assays done on different days with various samples. The variation from day to day was small.

Assay of milk and colostrum. Little variation has been found in the titres obtained with various samples of bulk milk and milk from cows in mid-lactation, but with colostrum the titres were generally much lower. Table 5 shows the results obtained with milk from cows before, during and after calving.

Discussion. With milk clear end-points were obtained, the tubes of inhibited cultures being purple, while the others were yellow and clotted. Occasionally with milk and always with colostrum, such clear-cut results were not obtained. Instead, with increasing dilution, there was a gradual decrease in the inhibition. This 'partial inhibition', manifested by the delay in coagulation, may extend with colostrum to high dilutions (Table 6).

This response to colostrum agrees well with that obtained by the previous method (see Fig. 3). It confirms that the substances responsible for the inhibition in colostrum are probably different from those present in milk. It was possible that colostrum contained only one of these substances, which was responsible for the partial inhibition,

whereas milk contained them both, complete inhibition and sharp end-points resulting. This possibility was tested in the following experiments.

Further evidence for two inhibitory substances

Milk heated to 70° C. for 20 min. loses nearly all its inhibitory activity when tested by the dilution method (Table 7). If an equal part of colostrum, with a very low titre in lactenin, is added to such a milk, its activity is restored. Even a small proportion of colostrum (1/100) added to heat-treated milk gives an inhibition comparable to that of raw milk (Table 7).

Several experiments were done to determine the optimum ratio of heat-treated milk and colostrum for maximum inhibition. The results of one such experiment are shown in Table 8. In this table dilutions of colostrum are given in the left-hand vertical column, the dilutions of heat-treated milk are shown in the upper horizontal line. Table 8 shows that the milk by itself was almost inactive (bottom horizontal line). The colostrum was

Table 5. *The estimation of the total inhibitory action of milk and colostrum*

		Date of sampling					
		11. ii	13. ii	15. ii	16. ii	18. ii	20. ii
Pippin 11, calved 15. ii	RF	465	150	30	30	100	125
	LF	65	185	25	37	100	150
	RH	45	65	20	30	65	100
	LH	100	150	13	30	100	100
		Date of sampling					
		11. i	23. i	6. ii	20. ii	27. ii	29. ii
Valerie, dried off 16. i, calved 29. ii	RF	30	37	125	20	20	150
	LF	30	45	150	–	20	125
	RH	30	55	82	7	20	100
	LH	25	37	82	6	20	100

	Date of sampling				
	20. ii	28. ii	10. iii	2. iv	7. iv
Bulk milk	80	100	80	80	100

Figures are units of lactenin per ml. of samples.

active at 1/12 only (right hand vertical column). A combination of these two components showed, however, a high activity. For instance, the colostrum diluted to 1/2330 gave a complete inhibition when added to heated milk diluted to 1/3 and the milk diluted to 1/115 was completely inhibitory when supplemented with colostrum diluted 1/135 to 1/690.

The term 'lactenin' has already been applied to the inhibitory substance of milk (5). For the sake of clarity we have accepted this term and extended its meaning to cover the inhibitory substance of colostrum (lactenin 1 or L_1). L_1 is thus defined as the substance mainly occurring in colostrum and giving partial inhibition to high dilutions of the sample. When supplemented with heat-treated milk, L_1 causes in our dilution assay complete inhibition of the test organism. That fraction of lactenin which appears to survive in milk heated to 70° C. for 20 min. has been termed lactenin 2 or L_2.

The characterization of the L_1 and L_2 substances by their pH *and heat stabilities*

Technique. Whey was prepared from separated milk by bringing the pH to 4·6 with N/10-HCl and then centrifuging. Portions of this whey were adjusted to different

Table 6. *The difference of response given by milk and colostrum in the dilution assay*

	Dilutions in autoclaved milk												
	1/6	1/9	1/13	1/20	1/30	1/44	1/68	1/100	1/152	1/225	1/340	1/500	

Table 6. *The difference of response given by milk and colostrum in the dilution assay*

	Dilutions in autoclaved milk												
	1/6	1/9	1/13	1/20	1/30	1/44	1/68	1/100	1/152	1/225	1/340	1/500	0
Milk	−	−	−	−	−	−	−	−	+ + +	+ + + +	+ + + +	+ + + +	+ + + +
Colostrum	−	−	+	+	+ +	+ +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + + +	+ + + +

− =no growth of the test organism *Str. pyogenes*.
+ to + + + + =approximate extent of growth of the test organism in milk containing bromocresol purple, corresponding to the following pH values:
− = > 6·5; + =6·1 to 6·5; + + =5·7 to 6·1; + + + =5·3 to 5·7; + + + + = < 5·3.

Table 7. *The activity of mixtures of heat-treated milk and colostrum in the dilution assay*

	Dilutions in autoclaved milk												
	1/2	1/4	1/8	1/16	1/32	1/64	1/128	1/256	1/512	1/1024	1/2048	1/4096	0
Raw milk	−	−	−	−	−	−	+ + +	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +
Heat-treated milk	+ + +	+ + +	+ + +	+ + +	+ + +	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +
Colostrum	+	+ +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + + +
½ colostrum, ½ heat-treated milk	−	−	−	−	−	+	+ +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + + +
Heat-treated milk + 1/100 colostrum	−	−	−	−	−	+ + +	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +

− =no growth of the test organism.
+ to + + + + =approximate extent of growth of the test organism (see Table 6).

Table 8. *The synergic action of the L_1 inhibitory substance in colostrum and the L_2 inhibitory substance in heat-treated milk*

Dilutions of colostrum	Dilutions of heat-treated milk															
	1/2	1/3	1/4·5	1/6·7	1/10	1/15	1/22	1/34	1/50	1/76	1/115	1/170	1/252	1/378	1/567	0
1/12	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
1/18	−	−	−	−	−	−	−	−	−	+	+ +	+ +	+ +	+ +	+ + +	+ + +
1/27	−	−	−	−	−	−	−	−	−	+ +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +
1/40	−	−	−	−	−	−	−	−	−	+ +	+ +	+ + +	+ + +	+ + +	+ + +	+ + +
1/60	−	−	−	−	−	−	−	−	−	+	+ +	+ + +	+ + +	+ + +	+ + +	+ + +
1/91	−	−	−	−	−	−	−	−	−	−	+	+ + +	+ + +	+ + +	+ + +	+ + + +
1/135	−	−	−	−	−	−	−	−	−	−	−	+ + +	+ + +	+ + +	+ + +	+ + + +
1/205	−	−	−	−	−	−	−	−	−	−	−	+ + +	+ + +	+ + +	+ + +	+ + + +
1/307	−	−	−	−	−	−	−	−	−	−	−	+ + +	+ + +	+ + +	+ + + +	+ + + +
1/460	−	−	−	−	−	−	−	−	−	−	−	+ + +	+ + +	+ + +	+ + + +	+ + + +
1/690	−	−	−	−	−	−	−	−	−	−	−	+ + +	+ + +	+ + + +	+ + + +	+ + + +
1/1037	−	−	−	−	−	−	−	−	−	−	+	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +
1/1553	−	−	−	−	−	−	−	−	−	+ + +	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +
1/2330	−	−	−	+ +	+ +	+ +	+ + +	+ + +	+ + +	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +
1/3500	+	+ + +	+ + +	+ + + +	+ + +	+ + +	+ + +	+ + +	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +
1/5245	+	+ + +	+ + +	+ + + +	+ + +	+ + +	+ + +	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +
1/7860	+ +	+ + +	+ + +	+ + + +	+ + + +	+ + +	+ + +	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +
1/11800	+ + +	+ + +	+ + + +	+ + + +	+ + + +	+ + + +	+ + +	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +
0	+ +	+ + +	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +

− =no growth of the test-organism.
+ to + + + + =approximate extent of growth of the test organism (see Table 6).

pH values, varying from 4·5 to 10·0. The samples were heated at temperatures from 59 to 74° C. each for 20 min. and then brought back to neutrality before assay.

The content in L_1 and L_2 of each of the samples was tested as follows:

For the assay of L_1, milk heated at 70° C. was added to the diluent to give a final concentration of 1/3 (this ensured an excess of L_2).

For the assay of L_2, a colostrum, known to contain L_1 at high concentration, was added to the diluent to give a final concentration of 1/300.

Results. The titres obtained in these assays have been plotted against the corresponding pH values (Figs. 5, 6). The resulting pH-thermal-destruction curves which were obtained enable the substances L_1 and L_2 to be differentiated. The maximum stability of L_1 is at pH 6·0–6·5, whereas it is pH 7·0 for L_2. At 71° C. L_1 is completely destroyed at any pH, while L_2 retains 50% of its activity when heated to 71° C. at pH 7·0. The heating of milk to 70° C. at pH 7·0 for 20 min. therefore destroys L_1, leaving L_2 in relatively high concentration.

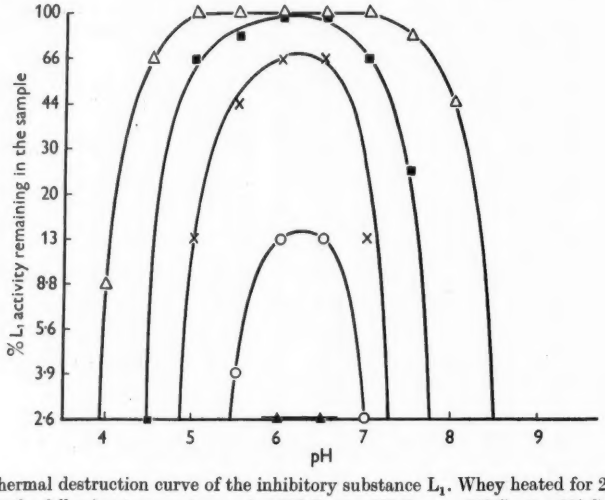

Fig. 5. The pH-thermal destruction curve of the inhibitory substance L_1. Whey heated for 20 min. at varying pH values, at the following temperatures: △ = 59° C., ■ = 62° C., × = 65° C., ○ = 68° C., ▲ = 71° C.

The separate estimation of L_1 and L_2 in unknown samples

Technique

Estimation of L_1. Samples were assayed by the dilution method, using in the diluent a constant concentration of milk heated at 70° C. for 20 min. (containing only L_2). This heat-treated milk must be in excess (generally one-third or one-quarter of the total volume). The sample itself received only the usual heat treatment of 58° C. for 20 min., leaving both substances L_1 and L_2 intact. To overcome the initial content of L_2 of the samples themselves relatively large concentrations of the L_2 supplement had to be used.

Estimation of L_2. The samples were first heated to 70° C. for 20 min. to destroy L_1. They were then assayed by the dilution method with colostrum at a constant concentration in the diluent. The colostrum, selected for its high content in L_1, was added to the medium at a low concentration (1/200 to 1/500).

For satisfactory assays, it was desirable to know the exact concentration at which to use the heat-treated milk or the colostrum, and the type of information shown in Table 8 was necessary for this. For instance, it was clear from such a table that, for the estimation

of L_2, the colostrum at concentrations of 1/500 was necessary, whereas for the estimation of L_1, heated milk had to be used at a concentration of 1/3.

The same standard as was used for the estimation of the overall activity was used in these assays. For the estimation of L_2 the standard was treated like the unknown, i.e. heated to 70° C. for 20 min. before assay.

Results. The results obtained with the method of assay for the separate estimation of L_1 and L_2 substances appeared to be within the limits of error of biological assays. Table 9

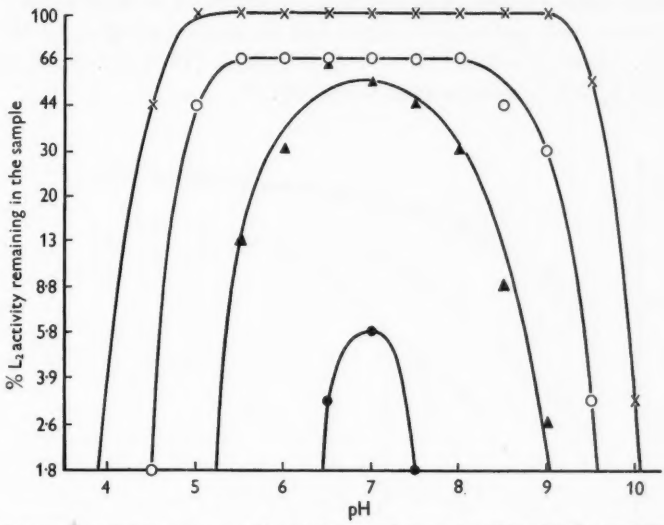

Fig. 6. The pH-thermal destruction curve of the inhibitory substance L_2. Whey heated for 20 min. at varying pH values, at the following temperatures: × =65° C., ○ =68° C., ▲ =71° C., ● =74° C.

Table 9. *The reproducibility of the assay for the separate estimation of L_1 and L_2*

	L_1 Replicates on three different days			L_2 Replicates on three different days		
	1	2	3	1	2	3
Ivy 2 (4 quarters), day before calving	1700	1500	1250	25	20	9
Ivy 2 (LF), day of calving	1700	1250	1250	20	6	—
Ivy 2 (4 quarters), 1st day after calving	83	150	125	35	45	—
Ivy 2 (RF), 3rd day after calving	100	150	190	125	125	—
Pippin 11 (4 quarters), 3rd day before calving	500	660	550	20	20	—
Bulk milk: M_1	100	125	—	80	65	—
M_5	100	100	—	55	65	—

Figures are units of L_1 or L_2 per ml. of sample.

gives the results of repeated assays on the same samples. The variations obtained were not excessive.

An example of the variation in the L_1 and L_2 content of cow's milk at the time of calving is given in Table 10. No such variation was observed with the bulk milk sample of a herd.

The effect of anaerobiosis and reducing agents on the inhibitory property of milk

Technique. Dilutions of milk were prepared as for an ordinary assay of the overall activity of milk, or for the separate estimation of L_1 or L_2. The tubes were then evacuated.

'Degassing' took about 1½ min., and the tubes were boiled for a further period of 1½–2 min. in a water-bath below 45° C. At the end of this time the tubes were sealed whilst under vacuum.

The degassing, followed by the boiling, was deemed sufficient to displace all gases with water vapour. This method of obtaining anaerobiosis was chosen because alkaline pyrogallol was found to be ineffective, and burning phosphorus, the only other safe method, might lead to a change in the pH of the media.

The reducing agent, cysteine, was also used, incorporated in the diluent.

Results. Anaerobiosis and cysteine which had no significant effect on the acid production of *Str. pyogenes* abolished the activity of lactenin (Table 11). Similar results were obtained when L_1 and L_2 were assayed separately.

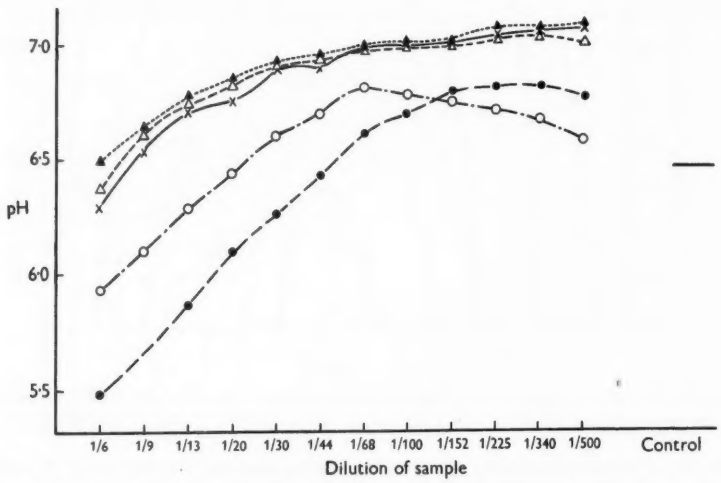

Fig. 7. The stimulatory effect of milk and colostrum on the acid production of *Str. agalactiae*. Dose-response curves obtained with : × = standard, ● = Ivy 2, one day before calving, ○ = same cow, the day of calving, △ = same cow, 1st day after calving, ▲ = same cow, 3rd day after calving.

Observations on the stimulatory properties of milk and colostrum

Milk is known to contain a variety of substances necessary for the growth of the more exacting micro-organisms. The concentration of these growth factors varies with the stage of lactation, the time of the year and the infection of the udder (30); the composition of colostrum differs markedly from that of milk, for example, the riboflavin content of colostrum may be about 10 times higher than that of milk (31).

Experiments to measure the growth-stimulating properties of milk or colostrum were made by diluting the samples in autoclaved milk, which was inoculated with *Str. agalactiae*, an organism known to be less sensitive to inhibition by milk than *Str. pyogenes*. After overnight incubation the resulting pH was measured. With small inocula the growth of *Str. agalactiae* is slow in autoclaved milk and, under these conditions, stimulation could be measured readily. This organism was therefore used in these tests. Peptone or other growth-stimulating supplements were not incorporated in the autoclaved milk because they masked the test.

Table 10. *The distribution of L_1 and L_2 in various samples of milk*

		Date of sampling					
		11. ii	13. ii	15. ii	16. ii	18. ii	20. ii
Pippin 11 (4 quarters), calved 15. ii	L_1	550	550	550	550	125	125
	L_2	20	20	6	6	55	65

		Date of sampling			
		11. ii	12. ii	13. ii	14. ii
Ivy 2 (4 quarters), calved 12. ii	L_1	1500	1500	125	150
	L_2	20	6	40	125

		Date of sampling		
		20. ii	10. iii	2. iv
Bulk milk	L_1	100	80	100
	L_2	65	37	60

Figures are units of L_1 and L_2 per ml. of sample.

Table 11. *The effect of anaerobiosis on lactenin*

		Dilutions of milk											
		1/2	1/4	1/8	1/16	1/32	1/64	1/128	1/256	1/512	1/1024	1/2048	0
Aerobiosis		−	−	−	−	−	−	−	+ + +	+ + + +	+ + + +	+ + + +	+ + + +
Anaerobiosis (quadruplicates)		+ + +	+ +	+ + +	+ +	+	+ +	+	+ + +	+ + +	+ + + +	+ + + +	+ + + +
		+	+	+ +	+ + +	+ + + +	+ + +	+ + + +	+ + +	+ + +	+ + + +	+ + + +	+ + + +
		(−)	+ + +	+ + +	+	(−)	+ +	+ + +	+ + +	+ + +	+ + + +	+ + + +	+ + + +
		+ + +	+	+ + +	(−)	+ +	+	+ + +	+ + +	+ + + +	+ + + +	+ + + +	+ + + +
Cysteine (mg. per 100 ml.)	10	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +
	1	−	−	−	−	+	+ +	+ + +	+ + +	+ + + +	+ + + +	+ + + +	+ + + +
	0·1	−	−	−	−	−	−	+	+ + +	+ + + +	+ + + +	+ + + +	+ + + +

− = no growth of the test organism.
+ to + + + + = approximate extent of growth of the test organism (see Table 6).
(−) = test organism fails to grow, probably because of inadequate anaerobiosis.

Fig. 7 shows the stimulatory effect of colostrum and milk. Colostrum was more stimulatory than milk, a finding confirmed with many samples. Whereas anaerobiosis abolished the inhibition, it did not affect the stimulatory action.

CONCLUSION

The experiments reported here show the complexity of the problem of the inhibition of micro-organisms by raw milk. Two substances, lactenin 1 and lactenin 2, have so far been found responsible for this inhibition, and methods are described for their estimation. They can only be separated by their different concentrations in milk and colostrum and by the small differences in their heat- and hydrogen-ion-stability. These two substances seem to be identical with the 'lactenin' of Jones, which was also to be found in whey, had about the same thermolability and was not dialysable. The influence of anaerobiosis and reducing agents also suggests that these substances are the same as studied by Wilson & Rosenblum (21).

The results of our experiments show that the inhibition given by raw milk is particularly dependent on the method used for its estimation. *Str. pyogenes* appears to be very sensitive to inhibition by raw milk when grown aerobically in milk containing peptone, but not when grown anaerobically. *Str. agalactiae* is also sensitive to inhibition by raw milk aerobically, but the addition of peptone enables this organism to overcome the inhibition. In fact, milk appears to be a poor medium for these organisms. *Str. pyogenes* grows much better in autoclaved milk supplemented with peptone and for *Str. agalactiae* the deficiency of sulphur compounds in milk is very marked. As will be shown in a later publication, cysteine, thioglycollic and thiomallic acids, sodium sulphide and even cystine, are very stimulatory to the growth of the latter organism in autoclaved milk. It is interesting that colostrum has a similar effect; it also enables *Str. agalactiae* to overcome the inhibitory power of raw milk.

The whole problem of the anti-streptococcal properties of milk appears to be linked with the nutritional requirements of the organisms used. Both inhibitory substances, L_1 and L_2, seem to interfere with the aerobic metabolism involving SH—groups. Wilson & Rosenblum (21) found that only the sulphur containing reducing agents inactivated lactenin, and, of the numerous 'reversing substances' they tried, only the sulphur containing vitamin thiamin had any effect.

We have obtained evidence, to be published in detail later, that lactenin is bacteriostatic rather than bactericidal. Cultures showing no growth after 48 hr. incubation were revived by out-diluting the inhibitory substances with suitable volumes of broth. Moreover, lactenin not only reduced the rate of growth, but reduced also the final weight of growth.

As Wilson & Rosenblum have pointed out (22), the anaerobic conditions of the udder make it unlikely that lactenin plays an important role in bovine mastitis. The growth-stimulating properties of milk are not, however, subject to this limitation. It is possible, therefore, that the growth of udder pathogens is more dependent on factors stimulating growth than on the inhibitory substances of milk.

SUMMARY

1. A diffusion cup technique using *Micrococcus lysodeikticus* has been found suitable for lysozyme estimation. By this method raw milk gave only small zones of inhibition. The inhibitory substance of milk was shown not to be lysozyme.

2. Dose-response curves (dilution *v.* pH) were obtained with *Streptococcus pyogenes* as the test organism. They indicated that the effect of milk on this organism was the result of the interaction of one stimulatory and at least two inhibitory substances.

3. The existence of two inhibitory substances was confirmed by dilution assays, one, L_1, occurring mainly in colostrum, the other, L_2, occurring mainly in milk. The inhibition is greatest when L_1 and L_2 act simultaneously.

4. The pH and heat stabilities of the two substances are slightly different. L_1 is most stable at pH 6–6·5, 90% being destroyed at 68° C. L_2 is most stable at pH 7 and 95% is destroyed at 74° C.

5. Methods for the separate estimation in unknown samples of L_1 and L_2 are described.

6. Anaerobiosis and cysteine abolish the inhibitory power of milk.

7. Milk and especially colostrum possess stimulatory properties which enable *Str. agalactiae* to overcome the inhibition.

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REFERENCES

(1) JONES, F. S. & LITTLE, R. B. (1927). *J. exp. Med.* **45**, 319.
(2) JONES, F. S. (1928). *J. exp. Med.* **47**, 877.
(3) JONES, F. S. (1928). *J. exp. Med.* **47**, 965.
(4) JONES, F. S. & SIMMS, H. S. (1929). *J. exp. Med.* **50**, 279.
(5) JONES, F. S. & SIMMS, H. S. (1930). *J. exp. Med.* **51**, 327.
(6) SINGH, K. & LAXMINARAYANA, H. (1948). *Ind. J. Dairy Sci.* **1**, 78.
(7) DAVIS, J. G. & MATTICK, A. T. R. (1936). *Agric. Progr.* **13**, 126.
(8) LEITCH, R. H. (1937). *Agric. Progr.* **14**, 40.
(9) DAVIS, J. G. & MCCLEMONT, J. (1939). *J. Dairy Res.* **10**, 94.
(10) THOMPSON, S. Y. (1945). Thesis, Reading University.
(11) ORLA-JENSEN, S. & JACOBSON, J. (1930). *Zbl. Bakt.*, Parasitenk., II. Abt. **80**, 321.
(12) ORLA-JENSEN, S. (1944). *Milchw. Zbl.* **73**, 1.
(13) HOBBS, B. C. (1939). *J. Dairy Res.* **10**, 35.
(14) MORRIS, C. S. & EDWARDS, M. A. (1949). *J. Dairy Res.* **16**, 161.
(15) MORRIS, C. S. & EDWARDS, M. A. (1950). *J. Dairy Res.* **17**, 253.
(16) PULLINGER, E. F. & KEMP, A. E. (1937). *J. Hyg., Camb.*, **37**, 527.
(17) ROSENAU, M. J. & MCCOY, G. W. (1908). *J. med. Res.* **18**, 165.
(18) HEINEMAN, P. G. (1903). *The Kinds of Bacteria concerned in the Souring of Milk.* Chicago.
(19) PIEN, J. (1952). *Lait*, **32**, 1.
(20) WILSON, A. T. & ROSENBLUM, H. (1952). *J. exp. Med.* **95**, 25.
(21) WILSON, A. T. & ROSENBLUM, H. (1952). *J. exp. Med.* **95**, 39.
(22) WILSON, A. T. & ROSENBLUM, H. (1952). *J. exp. Med.* **95**, 51.
(23) FOLLEY, E. J. & LEE, S. W. (1948). *Cornell. Vet.* **38**, 367.
(24) MCEWEN, A. D. & WHITE, M. B. (1950). *Vet. Rec.* **62**, 27.
(25) NEAVE, F. K., DODD, F. H. & HENRIQUES, E. (1950). *J. Dairy Res.* **17**, 37.
(26) HEATLEY, N. G. (1944). *Biochem. J.* **38**, 61.
(27) MCMORINE, H. G. & SLINN, G. S. (1948). *Canad. J. Publ. Hlth*, **39**, 203.
(28) THOMPSON, R. (1940). *Arch. Path.* **30**, 1096.
(29) FLEMING, A. (1932–33). *Proc. Roy. Soc. Med.* **71**.
(30) KON, S. K. (1951). *Ann. Nutr. Alim.* **5**, 211.
(31) HOUSTON, J., KON, S. K., THOMPSON, S. Y. & WHITE, P. (1940). *J. Dairy Res.* **11**, 145.

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500. THE SO-CALLED 'NASCENT' BACTERIOPHAGE PHENOMENON

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Several workers(1,2,3,4) have encountered a phenomenon in which a streptococcal bacteriophage, at the moment of its liberation from the host organism, appears to attack and lyse another streptococcal strain which is not normally susceptible to its action. Evans(1) applied the term 'nascent' to this specially potent form of phage, emphasizing that the term did not carry any implication as to the mechanism of the phenomenon.

Whitehead, Hunter & Cox(5) have reported that streptococcal phages, added to cultures in larger proportions than those commonly used for initiating lysis, have an inhibitory effect on certain strains which they cannot lyse. Under some experimental conditions the inhibitory effect might be confused with the lytic effect, although the fact that there is no increase in concentration of phage as a result of the action differentiates the two phenomena quite clearly. The capacity for inhibition is widespread among the phage races which attack strains of *Streptococcus cremoris* and is an indication of relationships between phages and organisms wider than those established by lytic action.

A further examination of several examples of inhibition by phage has shown that they adequately account for the 'nascent' phage phenomenon. The apparent lysing action of the newly formed phage on a heterologous strain of organism is actually an inhibitory action exerted by the high concentration of phage formed in the mixed culture by lysis of the homologous organism. An equivalent concentration of a stock preparation of phage can be shown to inhibit the heterologous organism. There is no need to postulate a special potency for 'nascent' phage.

MATERIALS AND METHODS

The cultures used were strains of *Str. cremoris* and one strain of *Str. lactis* which have been in use as cheese starters in New Zealand for several years. They were maintained in daily subculture in sterilized skim milk at 22° C. and were subjected to regular plating and repurification primarily for the purpose of maintaining their acid-producing activity. The phages, in the form of whey filtrates, were prepared as previously described(6) and were stored in the refrigerator at 5° C. Phage titres were determined by preparing a series of 1/10 dilutions of phage preparation in sterile saline and placing drops with a standard loop (0·004 ml.) on the surface of an agar plate previously spread with a mat of the susceptible culture. The titre was expressed as the highest dilution which gave plaques on the agar.

The action of a phage preparation on a strain of organism or on a mixture of strains was followed by inoculating tubes containing 9 ml. of sterilized skim milk with 2 drops (0·1 ml.) of a clotted 24 hr. milk culture of each strain and adding the desired amount of phage preparation. At intervals during incubation (usually at 30° C., but occasionally at 37° C., depending on the characteristics of the phage under trial) smears were made for

staining and examination under the microscope. The extent of growth was estimated by examination of a number of fields and was expressed by signs ranging from − to + + + +.

RESULTS

The observations of Anderson & Meanwell on the 'nascent' phenomenon were first confirmed in experiments in which *Str. lactis* (strain 756), supplied by them, was treated with a trace of its homologous phage (race 59) in the presence of either of our stock strains of *Str. cremoris* K or R_1. In the mixed cultures, K and R_1 both appeared to suffer lysis coincidently with 765, whereas neither K not R_1 in pure culture was affected by a trace of phage 59.

The experiments were extended to cover a selection of our stock strains of *Str. cremoris* and *Str. lactis* with the results detailed in Table 1.

Table 1. *Influence of phage* 59 *on strains of* Streptococcus cremoris *and* Streptococcus lactis *in the presence and absence of culture* 756

Titre of phage 10^{-7}.
0·05 ml. of 1/10 dilution added to 9 ml. milk.
Temperature of incubation 30° C.

Examined after (hr.)	Cultures: HP	K	R_1	R_6	ML_1	KH	E_3	ML_3
				Controls				
3	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +
5	+ + +	+ + +	+ + +	+ + +	+ + +	+ + + +	+ + +	+ + + +
				Cultures as above + phage 59				
3	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +
5	+ + +	+ + +	+ + +	+ + +	+ + +	+ + + +	+ + +	+ + + +
				Cultures as above + culture 756				
3	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +
5	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +
				Cultures as above + culture 756 + phage 59				
3	+	−	−	+ +	+ +	−	+ +	+ +
5	+	−	−	+	+ + +	−	+ + +	+ + + +

The results indicated that three of the cultures were apparently lysed by phage 59 in the 'nascent' state, two cultures were affected quite markedly but not completely destroyed (these cultures usually clotted the milk within 24 hours), and three cultures, including the one lactis strain ML_3, were not affected in their growth. Examination of the last series of tubes in the experiment after 5 hr. showed that all of them contained a high concentration of phage 59 but no trace of phage capable of lysing K, R_1 or KH cultures.

No progress was made towards an elucidation of the phenomenon until the inhibitory action of another phage race (51) on several of the cultures used in the experiment described above was observed (5). Even then a trial for inhibitory action by phage 59 did not give a positive result when 0·25 ml. of phage preparation was added to 9 ml. milk cultures of the organisms, a quantity which gave definite inhibition with preparations of phage 51 of similar titre. Finally, however, in an experiment in which culture K was added to a mixture of culture 756 and phage 59 at various times after the two had been in contact, it was made clear that K disappeared from the culture fluid in similar manner whether it was present while phage 59 was in the process of lysing culture 756 or long after lysis

had taken place. This indicated that the phage did not need to be in a 'nascent' state in order to act on K. The action was inhibitory just as in the case of phage 51, the difference being that phage 59 was somewhat less powerful in inhibitory action at a given titre.

A re-examination of cross-reactions between streptococcal strains and phage races disclosed several instances of inhibition of the type under discussion, and in each case it was possible under appropriate conditions to duplicate the 'nascent' phage phenomenon as originally described. An interesting example was provided by phage race 2 which lyses cremoris strain HP and inhibits lactis strain ML_3. Cultures HP and ML_3 could easily be distinguished from one another under the microscope; hence the extent of growth of each could be determined even in a mixed culture. Table 2 gives the results of an experiment in which phage 2 was (*a*) added in relatively large proportions to culture ML_3 alone in milk culture and (*b*) added in much smaller decreasing proportions to HP and ML_3 in mixed culture.

Table 2. *Influence of phage* 2 *on culture* ML_3 *in the presence and absence of HP*

Titre of phage 10^{-6}.
Temperature of incubation 30° C.

Volume (ml.) of phage added to 9 ml. milk culture

(*a*) Culture ML_3	Nil	0·05	0·1	0·25	0·5	1·0
3 hr.	+ +	+ +	+	+	−	−
5 hr.	+ + + +	+ + +	+	+	−	−

(*b*) Mixed cultures HP and ML_3 + 0·05 ml. of phage 2 in dilutions shown

	Controls		10^{-2}		10^{-3}		10^{-4}		10^{-5}		10^{-6}		10^{-7}	
	ML_3	HP	ML_3	HP	ML_3	HP	ML_3	HP	ML_3	HP	ML_3	HP	ML_3	HP
3 hr.	+ +	+ +	+	−	+	−	+	−	+ +	+	+ +	+ +	+ +	+ +
5 hr.	+ + + +	+ + +	−	−	−	−	+	−	+	−	+ +	−	+ + +	+ +

The results in the first part of the table indicate that phage 2 inhibited ML_3 strongly when present in any proportion exceeding 0·05–9 ml. of milk. The results in the second part of the table can be explained similarly on the basis of inhibitory action. Wherever the amount of phage 2 added to the mixed culture was sufficient to lyse HP in the early stages of the experiment and thus to form a high concentration of phage 2, growth of ML_3 was almost entirely prevented. If some growth of ML_3 had taken place before the high concentration of phage had been formed, it tended to be reduced. Generally, if ML_3 had reached more than a certain degree of growth before HP was lysed, the inhibition was very slight and temporary.

The extent of inhibition observed in any given instance of the phenomenon described above depends on several factors, viz.

(i) The inhibitory power characteristic of a given phage race on a given strain of organism.

(ii) The titre of phage produced in the mixed culture under the conditions of the experiment.

(iii) The stage of growth reached by the organism to be inhibited before the inhibitory phage is formed.

Instances of the effect of the first factor may be observed in Table 1. Culture HP and R_6 were less severely affected in the experiment detailed than were cultures K, R and KH. It was readily shown that this was due to the fact that HP and R_6 were inhibited to

a lesser degree by the direct action of phage 59 than were the other cultures. Cultures ML_1, E_8 and ML_3 were not affected by any concentration of phage 59 which it was practicable to apply. The results are similar to those obtained with phage 51 by Whitehead *et al.* (5), except that phage 51 showed in general a more powerful inhibitory action than phage 59.

The effect of the second factor may be observed whenever the proportion of phage added to a mixed culture is enough to lyse the sensitive organism so quickly that a high concentration of phage is not formed. The phage concentration finally present may then not be sufficiently great to give inhibition of the second organism. A mixed culture to which a lower proportion of phage was added initially may show more complete inhibition because it ultimately contains a higher phage concentration. An example of this is evident in Table 1 of Evans's paper (1), where the tube in which the largest proportion of phage was added to the mixed culture remained clear for several hours but became turbid overnight, the growth consisting of the strain which was more completely and permanently inhibited in the tubes to which smaller proportions of phage had originally been added. Whitehead *et al.* (5) noted with phage 51 that partial inhibition by a low concentration of phage was followed by recovery of the inhibited culture. Evans's result is consistent with the hypothesis that in the tube which showed a delayed growth, the organism sensitive to the phage was lysed too rapidly to permit a high titre to develop, and hence that the phage titre was ultimately lowest in the tube to which the largest proportion of phage had originally been added.

The effect of the third factor may be observed under a set of conditions where the strain inhibited by the phage is allowed to grow in the medium for some time before the phage is formed. These conditions may be created either by adding an extremely small inoculum of phage, as in the experiment detailed in the second part of Table 2, or by withholding both lysing culture and its homologous phage from the culture tubes until the culture to be inhibited has grown by itself for a period. In either set of circumstances it is then found that the phage when ultimately produced in high concentration can no longer so effectively check the growth of the strain it normally inhibits. This failure of inhibitory effect can readily be explained on the hypothesis that inhibition operates by attachment of one or more phage particles to each bacterium. Where the numbers of bacteria exceed the numbers of phage particles present, as may quite possibly be the case under some of our experimental conditions, the phage may exert its full effect and yet be unable to prevent the multiplication of some of the bacteria, with the result that there is merely a slight and temporary decrease in numbers as compared with a control culture.

DISCUSSION

Clarification of the mechanism of the 'nascent' phage phenomenon removes, in part at least, one unnecessary complication in the still very involved picture presented by phage-organism relationships in the group of lactic streptococci. The clarification is only partial because at present the exact mechanism of the inhibitory action is obscure. The picture presented under the microscope by a culture under the influence of an inhibitory phage is difficult to interpret. Provided that the concentration of phage is not too high (in which case no growth whatever occurs) the culture appears to commence growth, with the organisms increasing in number for 1 or 2 hr. A deterioration then sets in, and the cocci become noticeably irregular in shape and fewer in number. From this stage recovery may

occur with final clotting of the milk in 24–36 hr., or the organisms may disappear completely, depending on the proportion of phage present or possibly on the particular strain of organism under experiment. The appearances are perhaps better described as dissolution rather than as inhibition, and there is even a resemblance to lysis, although the process is more drawn out than in most instances of lysis. All observers agree that there is no multiplication of phage as a result of the process. The term inhibition is therefore used without any assumption as to the nature of the reaction.

From a practical dairying point of view the implications are but little changed by discarding the idea of 'nascent' phage and substituting the idea of inhibition by heterologous phage. Where two single strain starters are to be mixed in the cheese vat it is desirable that a phage which lyses one should not be capable of inhibiting the other. Inhibition will sometimes hamper acid production almost as much as lysis, but the effect depends to some extent on the stage of growth reached by the mixed culture before lysis of one of the strains present takes place.

SUMMARY

The apparent lytic effect of a newly formed ('nascent') streptococcal bacteriophage on streptococcal strains not normally lysed by the phage was shown to be actually an inhibitory action produced by high concentrations of the phage. The inhibitory effect was exhibited by stock preparations of the phage, used in sufficient concentration, and there was no need to postulate a special potency for the phage in its 'nascent' form. The inhibitory effect of phages on heterologous organisms was found to be a widespread phenomenon among strains of *Streptococcus cremoris* and *Str. lactis*. It became apparent only when larger proportions of phage than the traces normally used for lytic tests were employed.

REFERENCES

(1) Evans, A. C. (1940). *J. Bact.* **39**, 597.
(2) Anderson, E. B. & Meanwell, L. J. (1942). *J. Dairy Res.* **13**, 58.
(3) Hunter, G. J. E. (1943). *J. Dairy Res.* **13**, 136.
(4) Nichols, A. A. & Wolf, J. Z. (1945). *J. Dairy Res.* **14**, 81.
(5) Whitehead, H. R. Hunter, G. J. E. & Cox, G. A. (1952). *J. gen. Microbiol.* **6**, 21.
(6) Whitehead, H. R. & Hunter, G. J. E. (1939). *J. Dairy Res.* **10**, 403.

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501. THE EFFECT OF STORAGE AT DIFFERENT RELATIVE HUMIDITIES ON THE SURVIVAL OF MICRO-ORGANISMS IN MILK POWDER AND IN PURE CULTURES DRIED IN MILK

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(With 7 Figures)

The survival of the microflora during the storage of dried milks has been shown (1) to vary considerably from sample to sample even when the bacteria appeared to be of similar types and the milks were dried in the same plant. The variation may have been due to differences in the moisture content of the dried milk, but no data were available with which to test this hypothesis. Preliminary experiments with three different types of milk powder showed that over the limited range of humidity used, the lower the relative humidity in which the milk powders were stored, the greater the survival of the bacteria contained in the powder. The scope of the investigation was then extended to cover a wider range of relative humidity and longer periods of storage. It was found that although in general the higher the humidity (or moisture content) the lower the survival rate of the microflora in the milk powder, survival was greater at 5–20% relative humidity than at humidities above and below this level. A brief account of part of this work has been published elsewhere (2).

The fact that survival was greatest at 15–25% R.H. had been observed by Watts (3) for dried cultures of *Streptococcus agalactiae*. To determine whether this was a characteristic common to several types of bacteria the investigation was continued to include bacteria typical of the microflora of milk powders. Pure cultures of these bacteria in skim milk were dried and stored at varying relative humidities. Maximum survival was found at 5–20% R.H.

Dried suspensions of bacterial spores have been shown to be markedly sensitive to the complete absence of humidity, although their viability was unaffected by exposure to 5% R.H. for two years.

METHODS

Milk powders

Weighed portions of approximately 1 g. milk powder were spread in a thin layer in small Petri dishes (2 in. diam.) and placed in desiccators over aqueous sulphuric acid solutions of the specific gravities necessary to give the required relative humidities (4). The lids of the Petri dishes were raised slightly with small pieces of rubber tubing to allow free circulation of the air in the desiccator. At intervals two dishes were removed and immediately examined for moisture content and plate count. The moisture content was calculated as a percentage of the dry weight after heating at 100° C. for 3 hr.

The plate count, after reconstitution at room temperature, was made on Yeastrel milk agar at 37° C. and was calculated per g. of fresh powder. The relative numbers of

the different types of bacteria were determined by examining the colonies on the plates used for the count.

All experiments under controlled humidity were done in an incubator at 25° C.

Pure cultures

The cultures used were: (*a*) 0/3, a *Streptococcus* isolated from milk powder and similar to but not identical with *Str. faecalis*; (*b*) 0/7, an actively proteolytic *Micrococcus* frequently occurring in and isolated from milk powder; and (*c*) a typical strain of *Bacillus subtilis* isolated from raw milk.

(*a*) and (*b*) were grown in litmus milk and the cultures used before any appreciable acidity had been produced in the milk. One drop (32 drops per ml.) of an 18–20 hr. litmus milk culture was placed at the bottom of each of a series of small sterile tubes ($3 \times \frac{3}{8}$ in.). The tubes were plugged with cotton-wool, held over P_2O_5 for 2 days to remove most of the moisture and then placed in desiccators over suitable solutions to give the required relative humidities. At intervals, two tubes were removed from each desiccator, and the contents of each tube were reconstituted using a pipette and 9 ml. $\frac{1}{4}$-strength Ringer's solution to wash out the dried culture into a sterile tube. Plates were prepared from suitable dilutions, and the viable count was calculated per ml. of the original litmus milk culture. When the count fell to one colony per plate, i.e. one per ml. of reconstituted solution (equivalent to 271 per ml. litmus milk), the presence of any viable cells was determined by subculture to litmus milk.

The spore suspension of (*c*) in litmus milk was prepared as follows: growth from five 24 hr. milk agar slopes was suspended in 20 ml. $\frac{1}{4}$-strength Ringer's solution. This suspension was vigorously shaken by hand for about 5 min. and centrifuged at 2500 r.p.m. for 7 min. The supernatant liquid was heated at 60° C. for 90 min. to destroy the vegetative bacteria and then 0·15 ml. added to 15 ml. litmus milk. After vigorous shaking one drop of this spore suspension was placed in each of the tubes which were treated as described for (*a*) and (*b*). All observations were made after storage in an incubator at 25° C.

RESULTS

Milk powders

In preliminary experiments one sample of each of a spray-dried whole milk, a spray-dried separated milk and a roller-dried whole milk was stored for 8 weeks at relative humidities of 5, 30, 40 and 60%. The numbers of surviving bacteria gradually decreased with increase in relative humidity and hence with increasing moisture content of the powder (Table 1).

Two other samples of milk powder, F and M, were then held at 0, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100% R.H. for 48 and 103 weeks respectively. Sample F was a spray-dried separated milk with an initial moisture content of 5·07%, and with plate counts at 37 and 30° C. of 11,100 and 23,800 per g. respectively; it contained a mixed microflora. Sample M, a spray-dried whole milk from another plant, had a microflora consisting almost entirely of a few closely related types of spore-forming bacilli. The initial moisture content was 3·6 %, and the plate counts at 37 and 30° C. were 91,000 and 63,580 per g. respectively. Both samples were manufactured about 2 weeks before the beginning of the experiment.

The number of viable bacteria during the first 8–9 weeks of storage at all relative humidities are shown in Fig. 1 (sample F) and Fig. 2 (sample M). At the highest humidities,

90 and 100% R.H., the count first decreased and then increased rapidly. Growth of moulds began after 4 and 8 days at 90 and 100% R.H. respectively with sample F, and by that time the bacterial counts were more than 1 million per g. At 80% R.H. with sample F mould growth was first seen after 24 days, and the growth of the bacteria was slower than at 90 or 100% R.H.

With sample M moulding occurred after 5, 11 and 21 days at 100, 90 and 80% R.H. respectively. Bacterial growth had then begun but was much less marked than with

Table 1. *The percentage of viable bacteria* (37° C.) *in spray-dried whole and separated milks and in roller-dried whole milk stored for* 8 *weeks at different relative humidities*

Relative humidity (%)	Moisture content (%)		Viable bacteria (%)		
			Spray		
	Whole milk	Separated milk	Whole milk	Separated milk	Roller whole milk
60	5·4–6·0	7·7	5·2	2·6	2·6
40	5·1–5·3	7·3	12·0	43·2	38·9
30	3·6–3·7	4·7	27·7	54·5	67·1
5	1·6–1·9	2·1	74·5	81·0	94·5

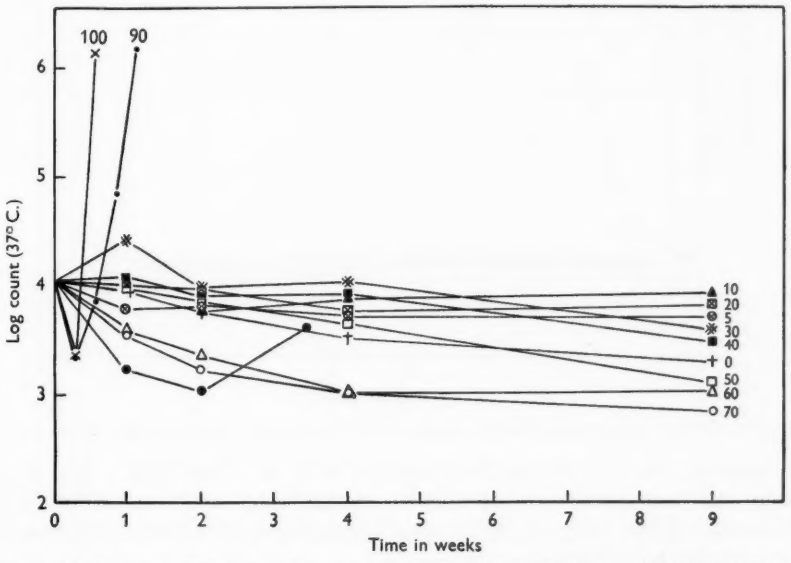

Fig. 1. The viable count in milk powder F when stored for 9 weeks at relative humidities from 0 to 100%. R.H. %: + 0, ⊗ 5, ▲ 10, ⊠ 20, ♯ 30, ■ 40, □ 50, △ 60, ○ 70, ● 80, • 90, × 100.

sample F, possibly due to the time taken for the germination of the spores before the multiplication of the vegetative forms of the bacteria. The main types of bacteria multiplying at 80–100% R.H. were micrococci in sample F and spore-forming bacilli in sample M.

With the exception of one dish of sample M at 70% R.H. in which one colony of a mould developed after storage for 70 weeks, no growth of moulds or bacteria occurred at relative humidities below 80%, that is, at moisture contents below 14·5% for sample F and 9·6% for sample M.

These figures agree closely with those obtained by Snow, Crichton & Wright (5), who found that moulding of food-stuffs stored at laboratory temperature did not normally take place below moisture contents, varying with the feed, from 9·8 to 15·7%.

The figures agree also with those of Van Dam & Warffemius (6) who studied several spray-dried separated milks during storage at high relative humidities. They found no moulding to occur during storage for 68 days at 75% R.H. when the moisture content of the eight samples examined varied from 9·8 to 14·5%. The time of storage observed by the Dutch workers before the onset of moulding was 2–3 weeks at 90 and 100% R.H. and 4 weeks at 85 and 80% R.H. These times were very much longer than those observed in the present work and also in earlier studies (7) on the storage of various feeding stuffs.

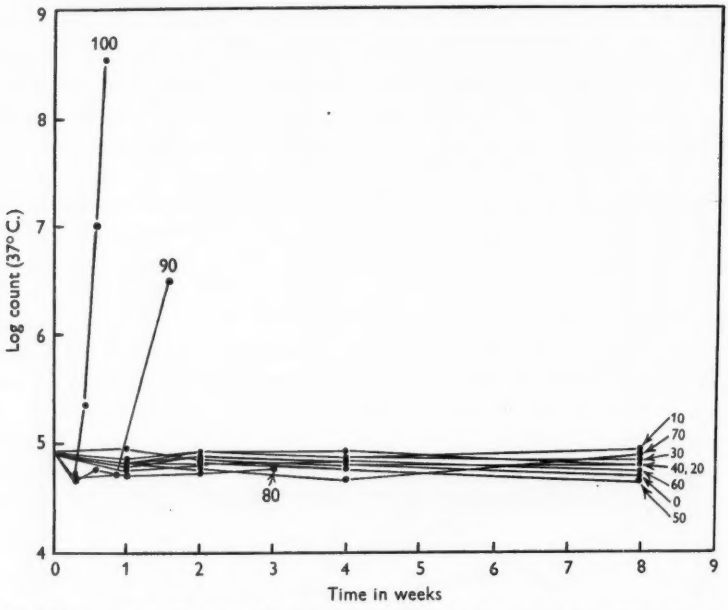

Fig. 2. The viable count in milk powder M when stored for 8 weeks at relative humidities from 0 to 100%.

This unusually slow rate of moulding appeared to be associated with a slow uptake of moisture. Gibbons & Fulton (8), on the other hand, found mould growth on dried egg containing 5–8% moisture, but their storage temperatures, 23·9 and 32·2° C., were higher and the relative humidity was not determined.

At relative humidities from 70 to 0%, the numbers of surviving bacteria decreased during storage. The percentage of surviving bacteria for the two milk powders at these humidities with the corresponding moisture content is given in Table 2. These figures show that with decreasing humidity the numbers of surviving bacteria increased to a maximum at 5–20% R.H. and then decreased again. The lower viability of the bacteria in the absence of moisture (0% R.H.) was particularly marked with sample M. This appears, as will be shown later, to be related to the high incidence of spore-forming bacteria in this sample.

When the time taken for the viable count at 37° C. to fall to a given percentage at each relative humidity was considered, it was found (Fig. 3) that the greatest number of

survivors in both F and M occurred in the range 5–20% R.H. Similar results were obtained for the viable count at 30° C., although for sample F the numbers and type of bacteria growing at 30 and 37° C. differed considerably.

The moisture content of the samples of dried milk showing maximum survival of the bacteria was 2·03–2·79% for the separated milk powder (F) and 2·04–3·10% for the whole-milk powder (M) (Table 2).

Table 2. *The percentage of viable bacteria* (37° C.) *after storage of milk powders* (*F and M*) *at relative humidities from* 0 *to* 70%

	Separated milk powder (F)		Whole milk powder (M)		
				Viable bacteria (%) after	
Relative humidity (%)	Moisture content (%)	Viable bacteria (%) after 48 weeks	Moisture content (%)	72 weeks	103 weeks
70	8·70	1	7·02	51	—
60	7·02	2	5·61	55	—
50	5·25	2	4·95	57	—
40	4·65	4	5·36	59	—
30	3·11	8	3·75	69	60
20	2·79	18	3·10	70	—
10	2·38	19	2·39	68	69
5	2·03	28	2·04	67	38
0	0·56	6	0·04	0·1	0·02

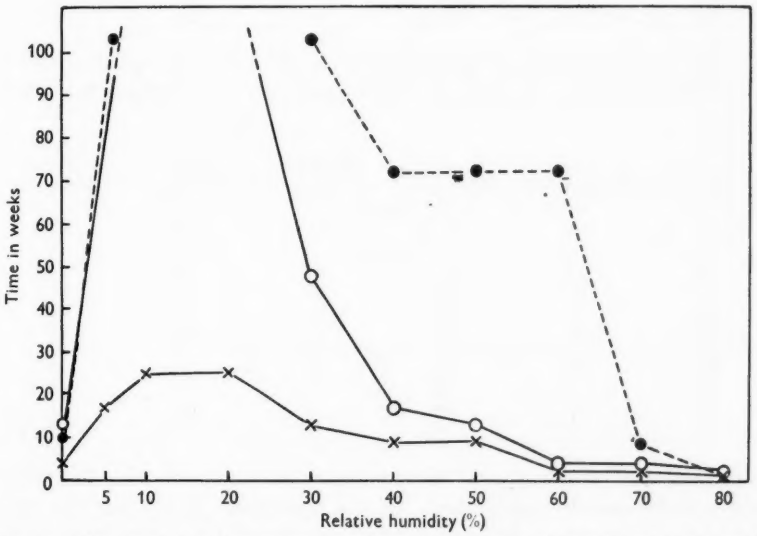

Fig. 3. The time taken to reach a given percentage of surviving bacteria when milk powders F and M were stored at different relative humidities. Separated milk (F), continuous lines: surviving bacteria (%): ○ 10, × 30. Whole milk (M), broken lines: surviving bacteria (%): ● 40.

Changes in the microflora of the milk powders during storage

The predominating types of micro-organisms surviving in the milk powders were determined at intervals by examining the colonies on the plates used for the counts. The percentage distribution of the microflora in sample F before and after storage for 48 weeks at relative humidities from 0 to 70% is given in Table 3 for the flora developing on plates

incubated at 37° C. and in Table 4 for that developing at 30° C. At relative humidities below 50 % the relative proportion of streptococci and micrococci growing on plates incubated at 37° C. showed little change, although the total number of surviving bacteria had fallen to 4–28 % of the original viable count. Although the proportion of micrococci and non-spore-forming rods on the plates incubated at 30° C. varied somewhat, these two main groups predominated below 40 % R.H. At relative humidities above 40–50 % the few surviving bacteria (less than 10 %) were predominantly spore-forming bacilli (37 and

Table 3. *Percentage distribution of the bacterial types* (37° C.) *in the separated milk powder* (*F*) *surviving storage for* 48 *weeks at relative humidities* (R.H.) *between* 0 *and* 70 %

R.H.	Streptococci	Micrococci	Rods: Non-spore-forming	Rods: Spore-forming	No. of surviving bacteria (%)
		Initial			
—	33	67	0	0	—
		48 weeks			
0	46	46	8	0	6
5	67	33	0	0	28
10	56	22	11	11	19
20	14	72	0	14	18
30	56	11	11	22	8
40	22	22	0	56	4
50	0	0	0	100	2
60	0	8	0	92	2
70	0	17	0	83	1

Table 4. *Percentage distribution of the bacterial types* (30° C.) *in the separated milk powder* (*F*) *surviving storage for* 48 *weeks at relative humidities* (R.H.) *between* 0 *and* 70 %

R.H.	Streptococci	Micrococci	Rods: Non-spore-forming	Rods: Spore-forming	No. of surviving bacteria (%)
		Initial			
—	0	50	50	0	—
		48 weeks			
0	10	10	80	0	11
5	10	50	40	0	39
10	0	20	80	0	40
20	0	42	58	0	27
30	0	90	10	0	19
40	0	20	40	40	6
50	0	1	0	67	2
60	0	40	0	60	1
70	0	0	0	100	1

30° C.), and on plates incubated at 37° C. the proportion of spore-forming bacilli increased gradually with decrease in viable count. On the other hand, at 0 % R.H. the number of surviving bacteria was also reduced to 6–11 %, yet the predominating types of bacteria showed little change from those in the fresh powder. The bacterial spores (or spore-forming bacilli) must then be at least as sensitive to the almost complete absence of moisture as the non-sporing bacilli and much more sensitive to the low than to the high relative humidities. Support for this conclusion was obtained with sample M in which the initial microflora consisted of about 20 % streptococci (*Streptococcus faecalis* group) and 80 % spore-forming rods of the *Bacillus subtilis* group. Because of the high proportion of spore-formers rather more than 50 % of the original microflora survived even after storage for 72 weeks at 70 % R.H., but at 0 % R.H. only 0·1 % of the bacteria were

still viable. Further confirmation was obtained with a pure culture of a spore-forming bacillus (p. 73).

The similarity in the relationships between the viability and the relative humidity shown by Watts (3) for a pure culture of *Streptoccocus agalactiae* and by the present author for the mixed microflora of commercially dried milk powders suggested that this might be a phenomenon common to most species of bacteria. It was therefore decided to extend the scope of the investigation to pure cultures of micro-organisms typical of the microflora of milk powders.

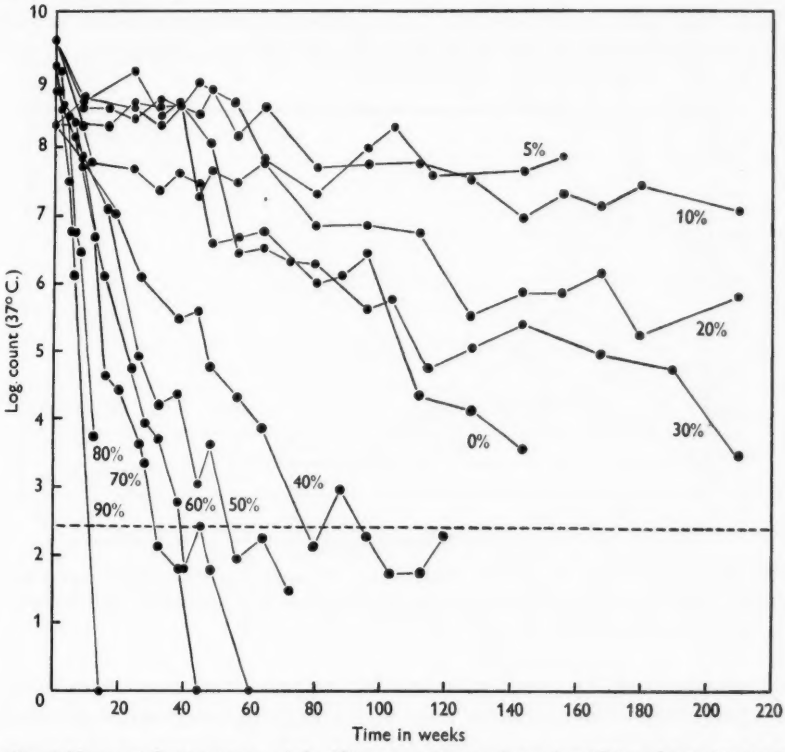

Fig. 4. The viable count during storage of the *Micrococcus* 0/7 at relative humidities of 0, 5, 10, 20, 30, 40; 50, 60, 70, 80 and 90% using sulphuric acid solution. The broken line represents the count per ml. when one colony per plate was obtained.

Pure cultures dried in milk

Micrococcus *and* Streptococcus

The decrease in the number of viable bacteria in a pure culture of the *Micrococcus* 0/7 which had been dried in litmus milk after storage at relative humidities from 0 to 90% (using aqueous sulphuric acid solutions) is shown in Fig. 4.

As with the milk powders there was a range of relative humidities between 5 and 20% within which maximum survival occurred (Fig. 5, solid lines). This is apparently a slightly lower range than that reported by Watts (3) (15–25%), but similar to that found for milk powders.

The specific gravity of the sulphuric acid when checked at the end of the 4-year period showed that there had been a rise of approximately 5% in the relative humidity in all desiccators originally at 0–40%. The lower limit of the range of maximum survival may therefore be nearer 10 than 5% R.H.

The *Streptococcus* 0/3 showed slightly longer survival times but otherwise behaved in the same way as the *Micrococcus*, and results for the *Streptococcus* have not therefore been presented.

A suggestion has been made (9) that the more concentrated solutions of sulphuric acid used to obtain low relative humidities may, in the presence of traces of organic matter, give rise to toxic gases which may affect the viability of the bacteria, but no evidence was presented in support of this suggestion. In the present investigation no trace of

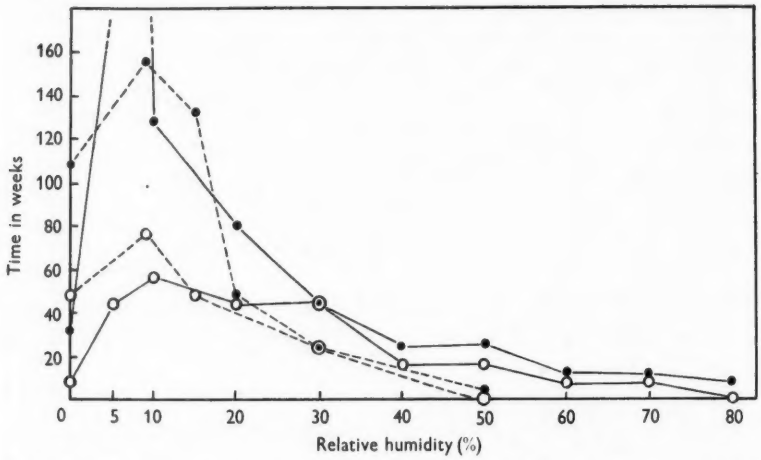

Fig. 5. The time taken to reduce the number of survivors to 10 and 1% when the *Micrococcus* 0/7 was stored at various relative humidities obtained by using either sulphuric acid or saturated salt solutions. H_2SO_4, continuous lines; salts, broken lines; surviving bacteria (%): ○ 10, ● 1.

sulphur dioxide could be detected by placing strips of filter-paper moistened with acidified potassium bichromate in the desiccators containing concentrated sulphuric acid (0% R.H.). In some of these desiccators a very thin white film formed on the inside of the chamber, and the outside of the tubes but did not appear to penetrate the cotton-wool plugs of the tubes. However, to avoid any possibility of misinterpretation of the results the experiments with 0/3 and 0/7 were repeated using other means of regulating the humidity as follows: 0% R.H. anhydrous $CaCl_2$ (after drying at 105° C. for 5 hr.); 9% R.H., phosphoric acid sp.gr. 1·75; 15, 20, 30 and 50% R.H., saturated solutions of $LiCl.2H_2O$, potassium acetate, $CaCl_2.6H_2O$ and $Ca(NO_3)_2.4H_2O$ respectively (10).

The decrease in the viable count during storage is given for the *Micrococcus* 0/7 in Fig. 6. The survival time for the cultures was rather less than in the previous experiment, but otherwise they behaved in the same way as when stored over the sulphuric acid solutions. Maximum survival occurred at 10% R.H. (Fig. 5, broken lines). Similar results were obtained with the *Streptococcus*. The validity of the sulphuric acid solutions as a non-toxic means of maintaining the required relative humidity was thus confirmed.

Spore-forming bacillus

The sensitivity of bacteria spores to the complete absence of moisture which was shown in the milk powders was confirmed with spores of a typical strain of *Bacillus subtilis*. The spore suspension dried in litmus milk was held at 0, 5, 10, 20 and 50% R.H. using sulphuric acid solutions and, as an alternative means of ensuring absence of moisture, over P_2O_5 and over anhydrous $CaCl_2$. No change in the numbers of viable

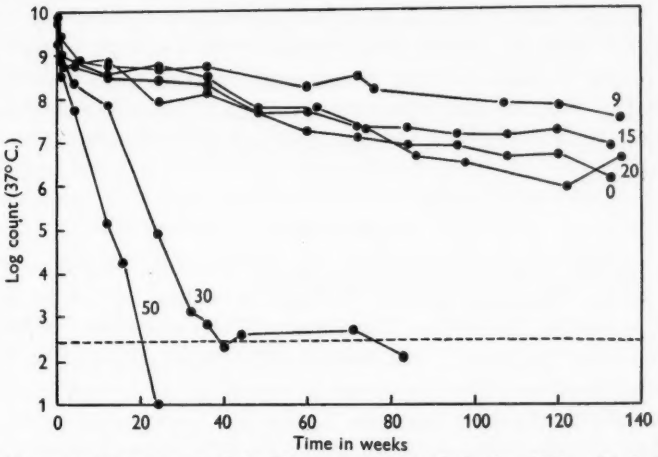

Fig. 6. The viable count during storage of the *Micrococcus* 0/7 at relative humidities of 0, 9, 15, 20, 30 and 50%. The broken line represents the count per ml. when one colony per plate is obtained.

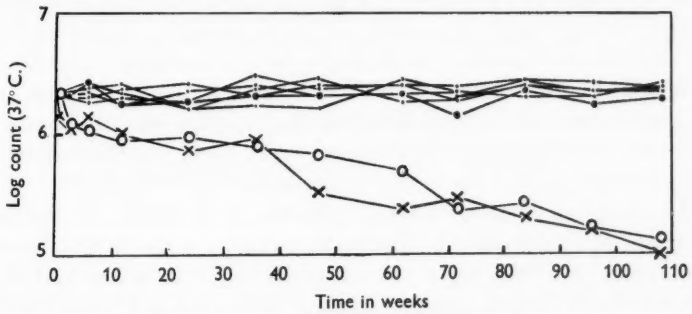

Fig. 7. The viable count of the spore suspension during storage at different humidities. × over P_2O_5; ○ over concentrated H_2SO_4; ● over solid $CaCl_2$; others at 5, 10, 20, 30 and 50% R.H.

spores took place during storage for 108 weeks at 5–50% R.H. or over $CaCl_2$ (Fig. 7). The desiccator containing $CaCl_2$ had been in use for humidity work for some time, and the results suggest that the humidity was now above 0%. No means of measurement of such low humidities was available to confirm this suggestion, but it may be noted that Johnson (11), using an Airguide humidity meter, reported a relative humidity as high as 7% over solid $CaCl_2$. Over P_2O_5 or concentrated H_2SO_4, however, the viability of the spores decreased slowly at an approximately steady rate, and after 108 weeks only 5% of the spores survived. Counts were normally made after incubation at 37° C. for only 24 hr. to prevent undue spreading and so facilitate counting of the colonies. Incubation for up to 5 days at 37° C. failed to show any sign of the development of additional

colonies and merely increased the error in counting due to the spreading of the existing colonies. One attempt to induce germination of the spores by mild heat shock was attempted after storage for 84 weeks over P_2O_5 and over concentrated H_2SO_4. Reconstitution of the samples was made in $\frac{1}{4}$-strength Ringer's solution preheated to 50° C., and the suspension was held at 50° C. for 15 min. before plating. No increase in the number of viable spores was shown when the results were compared in the usual method of reconstitution in the cold followed by the immediate plating of the suspension.

Spore suspensions which had been stored without loss of viability for 105 weeks at 10 and 50% R.H. showed a definite loss of viability (28 and 59% respectively) when stored for a further 6 weeks over P_2O_5. No material was available to test whether spores stored over P_2O_5 or concentrated H_2SO_4 would become viable once more if the moisture content was increased gradually by further storage at higher humidities.

DISCUSSION

It has been suggested that the difference in the survival of the microflora of dried milk in different samples during storage may be explained, at least in part, by differences in the moisture content of the powder. Confirmation of this suggestion has been obtained in the present work. Not only in milk powders but in pure cultures of bacteria dried in milk the rate of decrease in the viable count differed at different relative humidities and therefore at different moisture contents. During the storage of powders containing vegetative bacteria (*Bacterium aerogenes*), Bullock & Lightbown (12) found that, after an initial fall during the period required to attain equilibrium, the viable count fell logarithmically at a rate which was the same in a powder stored over P_2O_5 as when stored at 32% R.H. Using a wider range of humidity and longer periods of incubation, the present work has shown that the rates of fall in the viable count of vegetative bacteria at different relative humidities do differ, although the differences at 5, 10 and 20% R H., i.e. under conditions giving maximum survival, differ less than at other humidities including 0%. Although initially there may be a slightly more rapid fall in the viable count, the rate subsequently remains more or less steady at any given relative humidity.

Where the microflora consisted mainly of spores, as in sample M, changes in the moisture content had little effect on the viable count, and the maximum survival at 5–20% R.H. shown with this milk powder was probably due to the survival of the small number of vegetative bacteria present, although this could not be verified from the information available.

The sensitivity of bacterial spores, both in the milk powder M and in the dried spore suspension, to storage over P_2O_5 or concentrated H_2SO_4 was very striking. Bullock & Lightbown (12) failed to find any effect on the viability of spores of *Bacillus subtilis* spray-dried in peptone water and stored over P_2O_5 for 32 days. This apparent inconsistency with the present results may have been due to the greater resistance of the spores or to a greater protective effect of the different pabulum and to the shorter period of storage used by Bullock & Lightbown.

The question of dormancy or delayed germination of spores after storage under these conditions should not be overlooked. The spores may not be actually dead but lack viability under the normal conditions of resuspension and culture. Further investigation of this problem is proposed.

SUMMARY

1. Two spray-dried milks, one separated and one whole milk, have been stored at 25° C. for 48 and 103 weeks respectively at relative humidities ranging from 0 to 100%. At high humidities (80–100% R.H.) a rapid fall in the numbers of bacteria was followed by rapid growth of bacteria and by overgrowth by moulds. At relative humidities below 80% the number of surviving bacteria increased with decreasing humidity to maximum survival at about 10% R.H. and then tended to fall again towards 0% R.H.

2. The changes in the distribution of the different types of bacteria in the dried milks at different relative humidities are discussed.

3. Maximum survival at 5–15% R.H. has been confirmed with pure cultures (dried in milk) of a *Streptococcus* and a *Micrococcus* originally isolated from dried milks.

4. The marked loss of viability of bacterial spores noted in dried milks stored over P_2O_5 or concentrated H_2SO_4 has been confirmed for a dried suspension of the spores of a typical strain of *Bacillus subtilis*.

REFERENCES

(1) HIGGINBOTTOM, C. (1944). *J. Dairy Res.* **13**, 324.
(2) HIGGINBOTTOM, C. (1948). *Proc. Soc. appl. Bact.* p. 18.
(3) WATTS, P. S. (1945). *J. Path. Bact.* **57**, 191.
(4) WILSON, R. E. (1921). *J. industr. Engng Chem.* **8**, 326.
(5) SNOW, D., CRICHTON, M. H. G. & WRIGHT, N. C. (1944). *Ann. appl. Biol.* **31**, 111.
(6) VAN DAM, J. & WARFFEMIUS, G. G. (1944). *Leeuwenhoek ned. Tijdschr.* **10**, 123.
(7) SNOW, D., CRICHTON, M. H. G. & WRIGHT, N. C. (1944). *Ann. appl. Biol.* **31**, 102.
(8) GIBBONS, N. E. & FULTON, C. O. (1943). *Canad. J. Res.* **22**D, 332.
(9) CLAYTON, C. N. (1942). *Phytopathology*, **32**, 921.
(10) *Handbook of Chemistry and Physics* (1945). 29th ed. p. 1877. Ohio: Chemical Rubber Publishing Co.
(11) JOHNSON, L. P. V. (1946). *Canad. J. Res.* **24**C, 298.
(12) BULLOCK, K. & LIGHTBOWN, J. W. (1947). *Quart. J. Pharm.* **20**, 312.

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502. THE VITAMIN A POTENCY OF NEW ZEALAND BUTTER

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(With 5 Figures)

The vitamin A potency of New Zealand butter has not hitherto been extensively investigated. The values already reported in the literature are based mainly on analysis of occasional samples. They are of the same order of magnitude as the average values for northern European summer butters.

Malcolm & Pope (20), Herrick (10) and Barnicoat (2) have all published results from work carried out in New Zealand. Malcolm & Pope (20), by rat-feeding trials with factory butter samples from the Taranaki Province over the period February to July 1928, found that the vitamin A potencies of the winter butters were equal to or higher than those of the autumn butters. They attributed the higher potency of the winter butters to the lateness of the stage of lactation of the cows supplying the butter factories concerned. Herrick (10) reported results for vitamin A (estimated by the Carr-Price test and expressed in Lovibond 'blue units') in fats from samples of milk, cream and butter from the Manawatu district, but her results are of little value because the estimations were made on the unsaponified fat. Barnicoat (2) studied the variations in carotene content (Lovibond yellow values) and vitamin A content (Lovibond blue values) of some samples of butter from the Manawatu and Waikato districts during the 1935–6 dairying season. The average vitamin A potencies of the butters were: Manawatu 34 i.u./g., and Waikato 36 i.u./g. He also presented graphs showing seasonal variations in the carotene and vitamin A contents of butterfats from both districts. His results for Manawatu butters, however, were representative of only one herd; and the proportion of Jersey cows (55%) in this herd was considerably lower than that for the district as a whole (75–80%).

In England, Crawford, Perry & Zilva (7), by rat-feeding trials, found that the vitamin A potencies of samples of New Zealand butter made in the Taranaki Province in December 1927 and in May 1929 were almost as high as the maximum potency of the butters available to them from other sources. At Port Sunlight, in England, Morgan & Pritchard (22) also using the biological method, found the vitamin A potency of two New Zealand butters (previous history unknown) to be 24·0 and 23·4 i.u./g., compared with an average of 22·5 i.u./g. for seventy-five samples of European butter sampled at all seasons throughout the year.

The samples examined in the present survey were collected over a period of three years, from January 1946 to December 1948. Results of a similar survey to determine the seasonal variations and the variations from year to year in the vitamin A potency of milk and butter produced and marketed in different regions of the United States (5), and some results from Victorian butters (8), and for Swedish butters (29), have recently become available.

SAMPLING OF THE BUTTERS

Butterfat production in New Zealand is not evenly distributed over the various Provinces of the Dominion. Of the total amount of butterfat in cream and milk delivered annually to butter and cheese factories, the North Island supplies 91% and the South Island only 9%. Furthermore, of the butterfat produced in the North Island itself, a large proportion, amounting in the 1947–8 season to over 62% of the Dominion total, is produced in the Auckland Province (24). For the present survey* samples of creamery butter were obtained from twenty butter factories, selected in approximate relationship to the distribution of butterfat production over the Dominion and representing all the chief butter-making and also the chief cheese-making districts (see Table 1).

A 1 lb. pat of butter, taken at fortnightly intervals from a regular commercial churning at each of these factories, was forwarded to The Dairy Research Institute by post. The samples on arrival at the Institute were placed in storage at 10–20° F., and were held at this temperature for periods varying from 1 to 10 days, i.e. until required

Table 1. *Locality distribution of factories supplying samples for analysis*

Locality	No. of factories supplying samples
North Island:	
North Auckland (factories north of the City of Auckland)	4
South Auckland (including Franklin County, the Waikato, Thames Valley and the King Country)	5
Bay of Plenty	2
Gisborne	1
Hawkes Bay	1
Taranaki	2
Wellington (including Manawatu, Wairarapa and Pahiatua)	3
South Island:	
Canterbury	1
Southland	1

for analysis. At 8-weekly intervals two 4 lb. samples, taken from the same churning as the 1 lb. pat for the sampling date, were forwarded by each participating factory. These larger samples were held at 15° F. and were withdrawn for analysis after storage periods of 4 and 9 months respectively. The survey covers the analysis of 1517 samples of fresh butter, 335 samples of butter after 4 months' storage and 335 samples of butter after 8 months' storage.

Each sample analysed was representative of the butterfat from a large number of cows in the district supplying the factory. Some mixing of cream takes place in the neutralizing-vats; and the holding-vats in New Zealand butter factories have capacities of 1000–3000 gal. of cream, equivalent, when they are filled, to 5000–15,000 lb. of butter. The actual number of cows from which any individual sample originated, however, varied, for a number of reasons:

(1) From factory to factory with (*a*) variation in the size of the cream-holding vat used; (*b*) variation in the method of filling the cream-holding vats (at some factories the vats are filled simultaneously from the one header pipe); and (*c*) variation in the number of vats from which the churn was filled (at some factories cream may be drawn from

* Account was taken of butterfat in milk converted to cheese, since the survey was designed to provide also an indication of the vitamin A potency of New Zealand cheese.

several vats simultaneously for filling the churns, and even when this is not the case the churn may have been filled from the last portion of cream from one vat and the first draw-off from another vat).

(2) During the season with factories using more than one holding-vat, because of the variation in yield of butterfat per cow with progress of lactation and change of feeding conditions (it is common practice to utilize only the minimum number of vats required to contain the day's cream supply).

(3) At any one factory there could have been some variation in the locality within the district from which the cream supply for the particular churning sampled was drawn, owing to variation in the churning number sampled (vats are usually emptied in the same order each day).

Because of the operation of the above factors the numbers of cows represented by the sample of butterfat may have varied from 1000 to 25,000. It is well known that there are wide differences in the carotene and vitamin A contents of butterfats from different cows kept under the same feeding conditions. The number of cows represented by all the samples analysed during the investigation under review, however, was so great that it may be assumed that the effect of individual-cow variation was eliminated from the results, except that caused by a general variation in breed of cow from locality to locality (see discussion later).

EXPERIMENTAL

Preparation of sample for analysis. Butterfat was used in preference to butter (17). It was prepared by melting the butter sample, decanting the fat, and filtering at 60° C.

Method of analysis. The butterfat (8 g.) was saponified, the unsaponifiable matter was extracted with ether, and the ethereal extract was made up to 100 ml. Vitamin A was estimated in one portion (50 ml.) of the extract, by means of the Carr-Price test, according to the procedure already described (17). Carotene was estimated in the remaining 50 ml. of extract, by the following procedure:

The extract was carefully evaporated nearly to dryness in a water-bath at 50° C., and then to complete dryness under reduced pressure at room temperature. The residue was dissolved in 16 ml. of petrol ether (boiling point 40–60° C.), the solution was extracted twice with 90% methyl alcohol, to remove non-carotene pigments, and finally was washed, once only (4), with water. The solution was cleared, if necessary, by the addition of 2–3 drops of absolute alcohol, and the volume was made up to exactly 16 ml. with petrol ether. The yellow colour due to β-carotene was estimated in a Spekker absorptiometer, with use of violet filters O.B.I. giving maximum transmission at 440 mμ. The instrument was standardized against a solution of pure carotene (Eastman-Kodak) in petrol ether. Correction was not made for possible oxidative degradation products (11) nor for isomeric forms (42) of β-carotene.

In the discussion below the results for both carotene and vitamin A content are quoted mainly as micrograms per gram of butterfat. Where this course has not been followed, the units used are expressly stated.

RESULTS

(1) *Grouping of results according to main districts*

The results for both carotene and vitamin A contents of the butterfats from all the eighteen North Island factories for any one sampling date were fairly uniform, and those for the two South Island factories were uniform. The figures, therefore, have been assembled into five groups, respresenting the five main dairying districts, as follows:

District 1: North Auckland, results from four factories situated at Kaitaia, Whangarei, Ruawai and Waimauku.

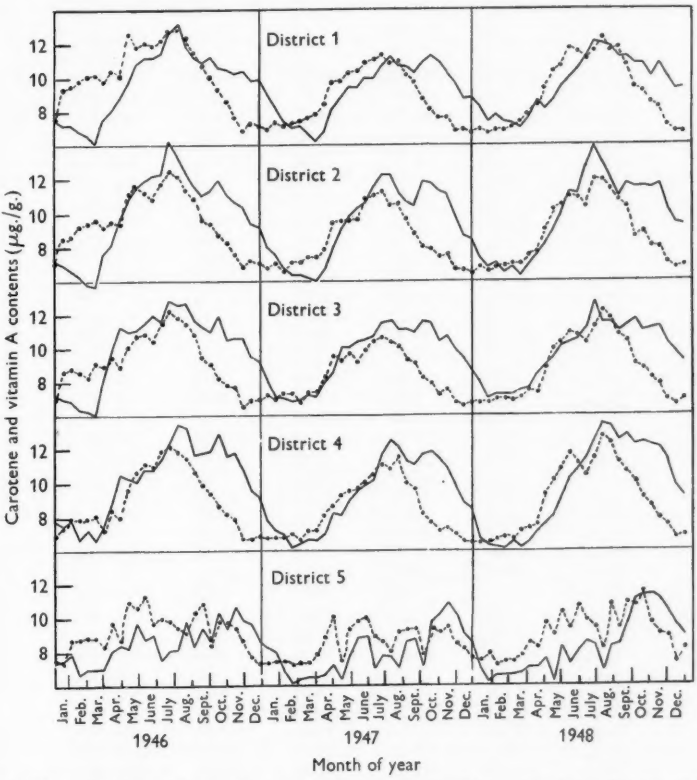

Fig. 1. Mean carotene and vitamin A contents of the butterfat samples for each main district for each fortnightly sampling date. — = carotene, -•-• = vitamin A.

District 2: South Auckland, results from five factories situated at Paerata, Morrinsville, Frankton, Otorohanga and Te Aroha.

District 3: Bay of Plenty, Gisborne and Hawkes Bay, results from four factories situated at Te Puke, Whakatane, Gisborne and Hastings.

District 4: Taranaki, and Wellington, results from five factories situated at Waitara, Hawera, Longburn, Pahiatua and Featherston.

District 5: South Island, results from two factories situated at Christchurch and Invercargill.

(2) *Averages of analytical results for butterfats in each district*

The carotene and vitamin A contents of the butterfats analysed, averaged for each main district for each fortnightly sampling date over the 3-year period, are shown in Fig. 1, and the average monthly results are shown in Tables 2A and 2B (see also unweighted mean annual results in Table 4 and statistical examination of these district results, and of results for individual factories, in Table 5).

Table 2A. *Average carotene contents of the butterfat samples from each main district for each month of the survey* (compare also Table 2B)

Year	Month	District 1 (μg./g.)	District 2 (μg./g.)	District 3 (μg./g.)	District 4 (μg./g.)	Range of North Island individual factory values (μg./g.)	District 5 (μg./g.)
1946	Jan.	7·22	7·11	7·20	7·72	6·5– 8·4	7·48
	Feb.	7·02	6·47	6·76	7·15	5·6– 8·9	7·41
	Mar.	6·40	5·78	6·08	7·10	5·4– 7·7	6·72
	Apr.	8·13	8·53	9·77	9·00	7·0–12·5	7·55
	May	10·27	11·38	10·88	10·33	8·5–12·7	8·68
	June	11·07	12·11	11·48	10·86	10·2–13·5	9·02
	July	12·04	13·55	12·06	12·13	11·0–15·2	8·01
	Aug.	12·59	12·99	12·25	13·45	11·3–14·9	9·06
	Sept.	11·14	11·42	10·89	11·78	9·8–12·8	9·76
	Oct.	10·34	11·62	11·09	12·41	9·7–13·5	10·47
	Nov.	10·19	10·60	10·51	11·27	9·2–12·5	10·44
	Dec.	9·78	9·40	9·34	9·55	8·5–10·6	8·81
1947	Jan.	8·50	7·83	7·76	7·73	6·4 9·2	7·49
	Feb.	7·19	6·63	7·09	6·90	5·3– 7·6	6·44
	Mar	6·66	6·25	6·95	6·68	5·7– 8·2	6·30
	Apr.	7·46	7·52	8·08	7·63	5·9–10·3	6·90
	May	9·28	9·58	9·90	8·69	7·6– 9·2	7·27
	June	9·61	10·61	10·19	9·84	8·6–11·6	9·19
	July	10·24	12·06	11·07	11·16	9·1–12·8	7·80
	Aug.	10·84	11·72	11·22	12·64	9·8–14·3	7·61
	Sept.	10·54	10·91	10·91	11·16	9·6–12·8	8·26
	Oct.	10·99	11·57	11·12	11·34	9·9–13·4	10·15
	Nov.	9·96	10·55	10·31	10·67	8·8–11·6	10·30
	Dec.	8·77	8·69	9·10	8·68	8·0–10·0	8·59
1948	Jan.	7·85	7·20	7·88	6·56	5·2– 8·7	6·41
	Feb.	7·64	6·88	7·56	6·41	5·0– 8·4	6·28
	Mar.	7·33	6·66	7·70	6·65	5·5– 8·6	6·42
	Apr.	8·31	7·76	8·76	7·50	6·2– 9·4	7·13
	May	8·92	9·23	9·61	8·84	6·4–11·2	7·20
	June	10·40	11·34	10·34	10·74	9·6–11·8	7·72
	July	11·49	13·44	12·02	12·20	9·9–15·0	8·37
	Aug.	11·71	11·61	10·73	13·21	10·0–15·3	8·23
	Sept.	10·97	11·56	11·05	12·37	10·5–13·2	10·30
	Oct.	10·52	11·51	10·80	12·21	9·7–13·4	11·31
	Nov.	10·19	10·93	10·65	11·54	9·5–12·6	10·58
	Dec.	9·24	9·45	9·54	9·50	8·6–10·2	8·75

(*a*) *Seasonal changes*

There was a very distinct and regular seasonal change in both the carotene and the vitamin A content of the butterfats from all the main districts (see Fig. 1, Tables 2A and 2B). The curves for the butterfats from *North Island districts* were remarkably similar in pattern, showing maximum and minimum at approximately the same time in each district each year, as shown in Table 3. The carotene and vitamin A contents showed a gradual and simultaneous rise during the late autumn and winter months. The fall in

carotene content during the midsummer period took place somewhat later than the fall in vitamin A content, and, as a consequence, the minimum in the vitamin A values persisted over a longer period than the minimum in carotene values.

Table 2B. *Average vitamin A contents of the butterfat samples from each main district for each month of the survey* (compare also Table 2A)

Year	Month	District 1 (μg./g.)	District 2 (μg./g.)	District 3 (μg./g.)	District 4 (μg./g.)	Range of North Island individual factory values (μg./g.)	District 5 (μg./g.)
1946	Jan.	8·21	7·85	7·82	7·34	6·5– 9·4	7·40
	Feb.	9·64	8·87	8·73	8·13	7·5–10·0	8·77
	Mar.	10·10	9·48	8·87	8·13	7·5–10·8	9·05
	Apr.	10·13	9·35	9·27	8·11	7·3–11·1	9·04
	May	12·26	11·18	10·91	10·15	9·0–12·9	11·35
	June	12·10	10·92	10·98	10·98	10·0–12·4	10·42
	July	12·46	12·11	12·14	12·05	11·0–12·9	10·16
	Aug.	12·56	11·62	11·63	11·59	10·9–13·0	10·21
	Sept.	10·81	9·86	10·01	10·28	8·0–11·8	10·60
	Oct.	8·96	9·39	8·42	8·59	6·9– 9·6	9·49
	Nov.	7·26	7·17	7·32	7·41	5·9– 8·4	9·02
	Dec.	7·10	7·12	7·11	6·77	6·1– 7·6	7·35
1947	Jan.	7·18	6·89	7·09	6·93	6·5– 7·4	7·38
	Feb.	7·24	6·85	7·36	6·94	6·0– 7·6	7·12
	Mar.	7·54	7·25	7·34	7·02	6·2– 7·9	7·44
	Apr.	9·06	8·66	8·95	8·39	7·6–10·0	9·52
	May	9·95	9·61	9·59	9·19	8·8–11·0	8·96
	June	10·56	10·13	9·78	9·81	8·7–11·4	10·00
	July	10·92	11·18	10·73	10·79	9·7–11·8	9·12
	Aug.	10·86	10·53	10·72	11·13	9·4–11·8	9·32
	Sept.	9·49	8·75	9·16	9·18	7·8–11·5	9·74
	Oct.	7·81	7·62	7·71	7·57	6·8– 8·5	8·89
	Nov.	7·13	7·10	7·33	7·14	6·5– 8·0	8·85
	Dec.	6·84	6·56	6·74	6·65	6·0– 7·1	7·64
1948	Jan.	6·80	6·75	6·65	6·54	6·2– 7·1	7·70
	Feb.	6·79	6·90	6·70	6·70	6·3– 7·2	7·36
	Mar.	7·31	7·15	7·03	7·03	6·3– 8·1	8·01
	Apr.	8·68	8·21	8·08	8·36	7·1– 9·7	9·11
	May	10·32	10·26	9·98	10·64	9·4–11·8	9·81
	June	11·34	10·71	10·94	11·28	9·8–12·2	10·25
	July	11·10	11·04	11·32	10·88	9·3–12·4	9·38
	Aug.	11·81	11·25	11·42	12·05	10·3–13·4	10·25
	Sept.	10·14	9·48	9·72	10·33	8·5–11·1	10·64
	Oct.	8·84	8·50	8·51	8·88	7·6– 9·3	10·27
	Nov.	7·66	7·61	7·42	7·67	6·9– 8·4	8·85
	Dec.	6·66	6·81	6·78	6·79	6·1– 7·1	7·70

Table 3. *Time of occurrence of maximum and minimum values for carotene and vitamin A contents of North and South Island butterfats*

	Constituent	Time of occurrence of: Maximum	Minimum
North Island	Carotene	July, August, September	February, March
	Vitamin A	June, July, August, September	November to March
South Island	Carotene	October and November	May to September
	Vitamin A	Winter or spring	November to March

The seasonal changes in carotene and vitamin A contents were less marked in *South Island* butterfats. The carotene content was at its maximum somewhat later in the season, and it remained at its maximum for a shorter time. The vitamin A content was at a maximum in winter, i.e. at the same time as for North Island butterfat (except in

1948, when the South Island maximum occurred in October); but the actual maximum values were lower and there were greater fluctuations in the results for consecutive samples (see Fig. 1) than applied in the results for North Island butterfat.

(*b*) *Range of variation of results for individual factories in the North Island*

The results in Tables 2A and 2B show that the range of variation in carotene and vitamin A contents of the fortnightly samples of butterfat at the individual factories was considerably greater than for the main district results. The grouping of the results into district values, therefore, has had the effect of making the carotene and vitamin A contents of the North Island butterfats appear more uniform than in actual fact they were. The greatest range of variation in the results for factory samples occurred in the late-autumn/winter/early-spring period.

(*c*) *Unweighted annual values—statistical examination of results*

The averaged results are given in Table 4, and a statistical examination of them and of all the original factory results for each sampling date is given in Table 5.

Table 4. *Unweighted mean carotene and vitamin A contents of butterfat samples for each district for each year of the survey*

	Mean carotene content for year (μg./g.)			Mean vitamin A content for year (μg./g)		
District no.	1946	1947	1948	1946	1947	1948
1	9·67	9·14	9·50	10·09	8·67	8·98
2	9·95	9·39	9·71	9·55	8·40	8·76
3	10·02	9·45	9·73	9·23	8·31	8·60
4	10·18	9·22	9·71	9·03	8·36	8·98
5	8·48	7·93	8·24	9·20	8·31	8·98

(i) *Carotene.* There were highly significant differences in the unweighted average carotene contents of the butterfats among the five districts in each of the three years of the survey. The average carotene result for district 5 was consistently lower than the corresponding results for the other four districts (see Table 4). A further statistical analysis on the results for districts 1–4 showed that in these North Island districts the differences in the annual averages for carotene content of the samples analysed were non-significant. The mean annual carotene values for each district were also compared by the *t*-test. The averages for district 5 were found to differ significantly, in each year, from those for all the other districts, but there was no significant difference among the mean results for the butterfats from districts 1–4, i.e. for North Island butterfats.

(ii) *Vitamin A.* There was a highly significant difference in the average annual vitamin A contents of the butterfat samples from the five districts in 1946 but not in 1947 and 1948 (see Table 5). The vitamin A contents of the butterfats from district 1 were abnormal during 1946, owing to the occurrence of a severe drought in the area. When the results for this district were eliminated from the analysis of variance, the differences in the average annual vitamin A content of the butterfat samples from the remaining four districts were non-significant. Thus, with this exception, the district averages for vitamin A content of the samples analysed did not differ significantly in any year of the survey.

(3) *Weighted mean monthly and annual carotene and vitamin A contents of the butterfat*

The quantities of butter produced in the separate dairying areas or subdistricts represented by each of the twenty factories were widely different; and, because of the seasonal nature of dairying in New Zealand, the quantity of butter represented by the fortnightly sample taken at any one factory varied throughout the year. For computation of the mean annual carotene and vitamin A contents of New Zealand butterfat, therefore, weighting of the results was necessary. The figures available for quantity of butter produced at each factory were on a calendar monthly basis. Hence: (*a*) the fortnightly results for the butterfats from the twenty factories were combined to give monthly results, representing averages of results for two, and sometimes three, sampling dates,

Table 5. *Analyses of variance of average carotene and vitamin A contents of butterfats from the five main districts for the years* 1946–8

Year	Component	Source of variance	Degrees of freedom	Sum of squares	Mean squares	Variance ratio	Significance
1946	Carotene (5 districts)	Between districts	4	117·33	29·33	6·15	Highly significant*
		Within districts	507	2416·64	4·77		
	Carotene, North Island districts	Between districts	3	14·75	4·92	—	Not significant
		Within districts	456	2334·64	5·12		
1947	Carotene (5 districts)	Between districts	4	92·45	23·11	6·47	Highly significant*
		Within districts	505	1800·74	3·57		
	Carotene, North Island districts	Between districts	3	6·41	2·14	—	Not significant
		Within districts	455	1698·05	3·73		
1948	Carotene (5 districts)	Between districts	4	98·44	24·61	5·03	Highly significant*
		Within districts	505	2470·12	4·89		
	Carotene North Island districts	Between districts	3	3·64	1·21	—	Not significant
		Within districts	454	2315·55	5·10		
1946	Vitamin A (5 districts)	Between districts	4	74·19	18·55	6·29	Highly significant*
		Within districts	507	1497·13	2·95		
	Vitamin A, districts 2, 3, 4, 5 only	Between districts	3	18·14	6·05	2·16	Not significant†
		Within districts	407	1140·29	2·80		
1947	Vitamin A (5 districts)	Between districts	4	8·24	2·06	—	Not significant
		Within districts	505	1225·19	2·43		
1948	Vitamin A (5 districts)	Between districts	4	7·41	1·85	—	Not significant
		Within districts	505	1892·57	3·75		

* 5% level 2·37, 1% level 3·32.
† 5% level 2·60, 1% level 3·78.

and the butter production in the area surrounding the factory was computed from the figures for all the factories in the area (the twenty subdistricts included all the butter factories in the Dominion); (*b*) the figures computed as under (*a*) were combined to give monthly butter productions and monthly weighted mean carotene and vitamin A results for the butterfats for each of the five main districts as set out in Table 1, for each month of the survey (Figs. 2A and 2B); (*c*) the weighted monthly results for the butterfats for the three years were combined to give weighted monthly results for North Island and South Island butterfat (Tables 6A and 6B, Fig. 3); and (*d*) the figures computed as under (*b*) were combined to give weighted annual butter productions and weighted annual carotene and vitamin A results for butterfats for the five main districts and for New Zealand as a whole (Table 7).

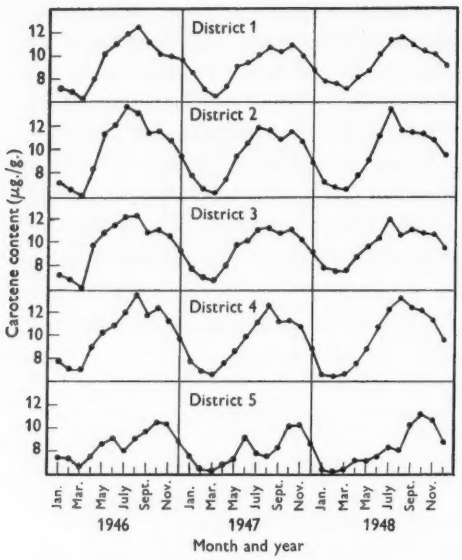

Fig. 2A.

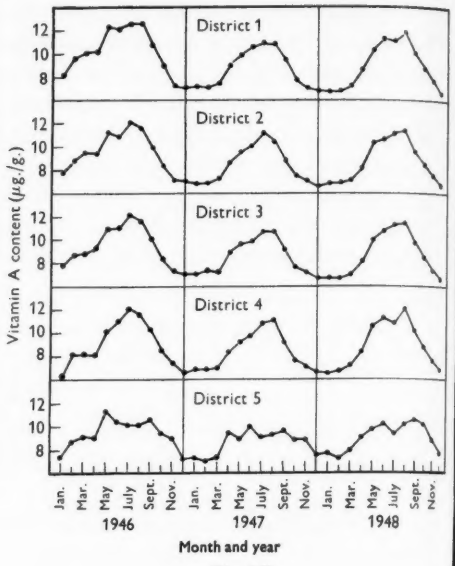

Fig. 2B.

Fig. 2A. Weighted mean carotene contents of butterfat from each main district for each month of the survey.
Fig. 2B. Weighted mean vitamin A contents of butterfat from each main district for each month of the survey.

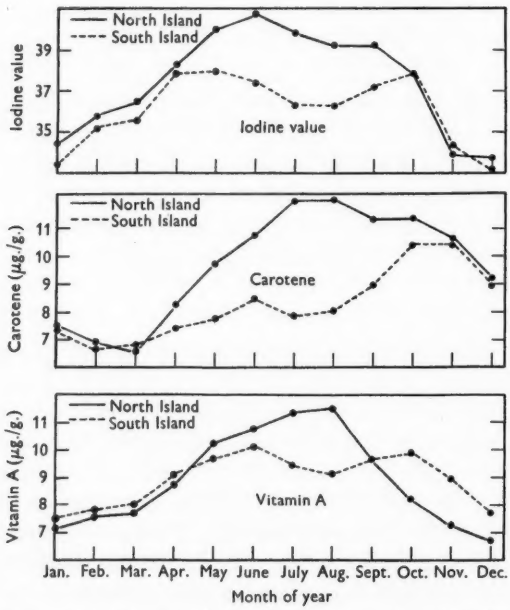

Fig. 3. Weighted mean monthly carotene and vitamin A contents and monthly iodine values of North Island and South Island butterfats.

(i) *Mean monthly results for North Island and South Island butterfat*

The mean monthly *carotene* content of North Island butterfat was higher throughout the season than that of South Island butterfat (Table 6A and Fig. 3), but, on the other hand, the mean monthly *vitamin A* content of North Island butterfat was for a considerable proportion of the year lower than that of South Island butterfat (Table 6B, Fig. 3).

Table 6A. *Weighted mean monthly carotene contents of North Island and South Island butterfats over the three years of the survey*

	Carotene (μg./g.)			
	North Island		South Island	
Month	Mean	Range of monthly district means	Mean	Range of monthly district means
Jan.	7·53	6·56– 8·50	7·14	6·41– 7·49
Feb.	6·91	6·41– 7·64	6·70	6·28– 7·41
Mar.	6·63	5·78– 7·70	6·48	6·30– 6·72
Apr.	8·06	7·46– 9·77	7·21	6·90– 7·55
May	9·69	8·69–11·38	7·79	7·20– 8·68
June	10·80	9·61–12·11	8·64	7·72– 9·19
July	12·39	10·24–13·55	8·06	7·80– 8·37
Aug.	12·06	10·73–13·45	8·34	7·61– 9·06
Sept.	11·26	10·54–12·37	9·52	8·26–10·30
Oct.	11·37	10·34–12·41	10·67	10·15–11·31
Nov.	10·65	9·96–11·54	10·45	10·30–10·58
Dec.	9·22	8·68– 9·78	8·72	8·59– 8·81
All months	9·54	5·78–13·55	8·41	6·28–11·31

Table 6B. *Weighted mean monthly vitamin A contents of North Island and South Island butterfats over the three years of the survey*

	Vitamin A (μg./g.)			
	North Island		South Island	
Month	Mean	Range of monthly district means	Mean	Range of monthly district means
Jan.	7·11	6·41– 8·21	7·49	7·38– 7·70
Feb.	7·37	6·70– 9·64	7·74	7·12– 8·77
Mar.	7·64	7·02–10·10	8·17	7·44– 9·05
Apr.	8·64	8·08–10·13	9·21	9·04– 9·52
May	10·10	9·19–12·26	10·14	8·96–11·35
June	10·64	9·78–12·10	10·23	10·00–10·25
July	11·40	10·73–12·46	9·59	9·12–10·16
Aug.	11·30	10·53–12·56	9·98	9·32–10·25
Sept.	9·63	8·75–10·81	10·36	9·74–10·64
Oct.	8·27	7·57– 8·96	9·57	8·89–10·27
Nov.	7·34	7·10– 7·67	9·58	8·85– 9·02
Dec.	6·82	6·56– 7·12	7·57	7·35– 7·70
All months	8·12	6·41–12·56	8·57	7·12–10·64

(ii) *Mean main district annual results*

The mean annual carotene and vitamin A contents of the butterfats from the four North Island districts for any one year were very uniform (Table 7); but there were some variations from year to year—the carotene results were consistently lower in 1947 than in 1946 and 1948, and the vitamin A results were lowest in 1947 and highest in 1946. The results for both carotene and vitamin A contents of the butterfats for district 5 were fairly uniform from year to year over the three years of the survey.

(iii) *Mean North Island and South Island annual results*

The mean annual carotene contents of butterfat from the North Island districts for the three separate years of the survey were consistently higher than those for South Island butterfat (Table 7); as could be expected from the comparisons in Figs. 1, 2A and 3. The weighted mean annual carotene contents for North Island and South Island butterfats for the three years of the survey were 9·54 and 8·41 μg./g. respectively (Table 6A).

The mean annual vitamin A content of North Island butterfat, on the other hand, was consistently lower than the corresponding South Island figure. The weighted mean annual vitamin A contents of North Island and South Island butterfats for the three years of the survey were 8·12 and 8·57 μg./g., respectively. The difference in the mean annual vitamin A contents of the butterfats for the two islands is rather greater than would be

Table 7. *Weighted mean annual carotene and vitamin A contents for butterfats from each district*

		Carotene (μg./g.)		Vitamin A (μg./g.)	
Year	District	Mean	Range of monthly means	Mean	Range of monthly means
1946	1	9·57	6·40–12·59	8·80	7·10–12·56
	2	9·90	5·78–13·55	8·60	7·12–12·11
	3	9·62	6·08–12·25	8·59	7·11–12·14
	4	9·90	7·10–13·45	8·39	6·77–12·05
	5	8·63	6·72–10·47	8·83	7·35–11·35
	All districts	9·71	5·78–13·55	8·61	6·77–12·56
1947	1	9·18	6·66–10·99	7·85	6·84–10·92
	2	9·33	6·25–12·06	7·74	6·56–11·18
	3	9·31	6·95–11·22	7·88	6·74–10·73
	4	9·21	6·68–12·64	7·68	6·65–11·13
	5	8·17	6·30–10·30	8·22	7·12–10·00
	All districts	9·20	6·25–12·64	7·80	6·56–11·18
1948	1	9·35	7·33–11·71	8·08	6·66–11·81
	2	9·63	6·66–13·44	8·05	6·75–11·25
	3	9·57	7·56–12·02	7·98	6·65–11·42
	4	9·72	6·41–13·21	8·25	6·54–12·05
	5	8·42	6·28–11·31	8·66	7·36–10·64
	All districts	9·50	6·28–13·44	8·12	6·54–12·05
Dominion average 1946–8		9·45		8·15	

expected from a study of the lines for vitamin A content in Figs. 1 and 3 and of the results for analysis of variance in Table 5. The explanation lies in the fact that it is during the winter period of low production that the North Island values are higher than the corresponding South Island values, and that during the summer period of high production the values are lower (see also discussion, later, on effect of breed of cow on carotenoid content of butterfat).

(iv) *Weighted results for New Zealand butterfat*

The weighted New Zealand results for the three separate years of the survey and for the three years combined are given in Table 7. The carotene results showed a smaller variation from year to year than the vitamin A results, but both carotene and vitamin A results were lowest in 1947. The mean carotene and vitamin A contents of New Zealand butterfat over the three-year period were 9·45 and 8·15 μg./g. respectively.

(4) *Vitamin A potency of the butterfat*

The results presented above have been expressed in terms of micrograms of carotene and vitamin A per gram of butterfat. For the calculation of the potency values in terms of international units of vitamin A per gram of butter 1 μg. of carotene has been taken as the equivalent of 1·67 i.u. and 1 μg. of vitamin A as the equivalent of 3·33 i.u., in accordance with the recommendations of the Expert Committee on Biological Standardization of the World Health Organization (41). It has been assumed, too, that New Zealand butter contains 82·0% of butterfat. American workers (5), in calculating the vitamin A potencies of butters from their analytical figures, have applied a correction on the basis of their finding that when vitamin A was added to the butterfat before analysis only 93% of the quantity added was recovered. In the procedure for vitamin A estimation used in the present investigation there was a similar 7% loss of added vitamin A. The figures available for vitamin A content and potency of butterfat in other countries, however, usually do not include allowance for this correction; and in the present paper the results for vitamin A content of butterfat quoted as micrograms per *gram* are, with one exception, actual recorded values uncorrected for loss in estimation, and the potencies are given as derived from the uncorrected values. The one exception is Table 9, where corrected values also are quoted to facilitate comparison of the New Zealand figures with those for other countries where only the corrected values are quoted.

(*a*) *Monthly potency values*

The weighted average vitamin A potency of New Zealand butter for the 36 months of the survey are given in Table 8, and the average monthly potencies for the three years are shown in Fig. 4. The combined seasonal trends in the carotene and vitamin A contents of the butterfats (see above) are reflected in the variations in the potency of the butter, giving sharper changes at both the maximum and the minimum. The highest potencies, viz. 45·0–50·2 i.u./g., were found in the winter butters (July and August) and the lowest potencies, viz. 28·3–33·4 i.u./g., in the summer and early autumn butters (January, February, March). The potency of the butter was falling to a minimum when the butter production was at a maximum, and the potency was approaching its maximum value when the butter production was at a minimum. This occurrence of the near-minimum potency values at the season of maximum butter production causes the mean potency value for New Zealand butter (i.e. weighted for variation in monthly butter production) to be considerably lower than would be expected from a casual study of the analytical figures.

(*b*) *Annual potency values*

The results for annual potency are given in Table 9. The mean vitamin A potency of New Zealand butter over the *three-year period** was 35·2 i.u./g. ((uncorr.) or 36·9 i.u./g. (corr.), of which 12·9 i.u. were contributed by the carotene and 22·3 i.u. (uncorr.) or 24·0 (corr.) by the vitamin A. The mean vitamin A potency of New Zealand butter, 35·2 i.u./g. (uncorr.), is equivalent to a potency of 15,970 (or, approximately 16,000) i.u. per pound. The values (uncorr.) obtained by Morton (23) by spectrophotometric estimation on samples of New Zealand butter analysed in England during the years 1939–44,

* The annual butter productions for the three years were: 1946, 282,149,000 lb.; 1947, 341,028,000 lb.; 1948, 342,950,000 lb.

viz. 12 i.u./g., contributed by carotene and 24 i.u./g. contributed by vitamin A, are thus in close agreement with those obtained in the present much more extensive investigation.

(*c*) *Summer and winter potency values*

In surveys of vitamin A potency of butter published elsewhere it has been customary to quote weighted mean potencies for 'winter' and 'summer' butters. The results of

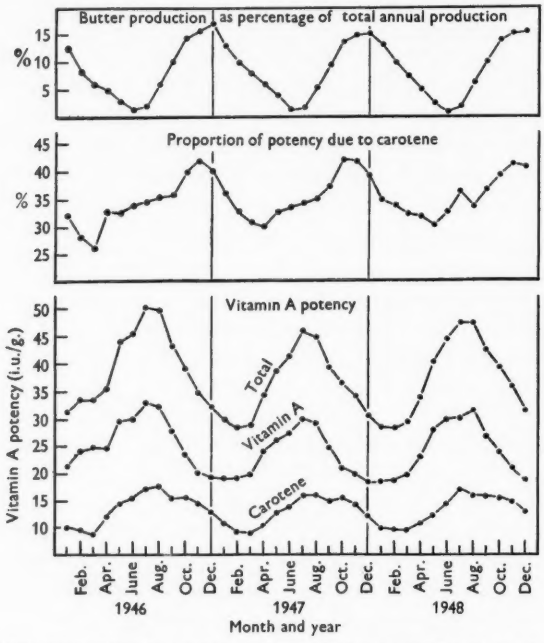

Fig. 4. Weighted mean vitamin A potency of New Zealand butterfat related to percentage butter production monthly over three years, 1946–8.

a similar grouping of the potencies (uncorr.) found for New Zealand butter in the present investigation are as follows:

Winter butter: weighted means for butters made during April to August (inclusive), representing 17% of the average annual production	41·9 i.u./g. 19,010 i.u./lb.
Summer butter: weighted means for butters made during September to March (inclusive), representing 83% of the average annual production	33·8 i.u./g. 15,330 i.u./lb.

(*d*) *Comparison of vitamin A potency of New Zealand butter with potencies of butter in overseas countries*

Some comparative figures are given in Table 10. The results available for United States butters (5) are not directly comparable with those given in Table 10, because they are based on use of the factor 4·0 instead of the now internationally accepted 3·33 for conversion of vitamin A to international units, and because they are corrected for the

Table 8. *Weighted mean monthly vitamin A potencies of New Zealand butter over the period* 1946–8

Year	Month	Monthly butter production as percentage annual production (%)	Vitamin A potency due to		Total vitamin A potency (i.u./g.)	Proportion of total potency due to carotene (%)
			Carotene (i.u./g.)	Vitamin A (mean) (i.u./g.)		
1946	Jan.	12·4	10·0	21·2	31·2	32·1
	Feb.	8·0	9·4	24·0	33·4	28·2
	Mar.	6·1	8·8	24·7	33·5	26·2
	Apr.	4·9	11·9	24·5	36·4	32·7
	May	2·9	14·4	29·6	44·0	32·6
	June	1·3	15·4	30·0	45·4	33·8
	July	1·8	17·3	32·9	50·2	34·5
	Aug.	5·9	17·6	32·0	49·6	35·4
	Sept.	10·1	15·4	27·7	43·1	35·8
	Oct.	14·3	15·6	23·5	39·1	39·9
	Nov.	15·6	14·5	20·2	34·7	41·9
	Dec.	16·8	12·9	19·3	32·2	40·1
	Mean*	—	13·3	23·5	36·8	36·1
1947	Jan.	12·7	10·8	19·1	29·9	36·1
	Feb.	9·7	9·3	19·2	28·5	32·8
	Mar.	7·9	8·9	19·9	28·8	30·9
	Apr.	5·8	10·3	24·0	34·3	30·1
	May	3·7	12·6	26·0	38·6	32·7
	June	1·3	13·9	27·5	41·4	33·5
	July	1·6	15·7	30·0	45·7	34·3
	Aug.	5·3	15·8	29·1	44·9	35·2
	Sept.	9·3	14·7	24·7	39·4	37·4
	Oct.	13·4	15·4	21·1	36·5	42·2
	Nov.	14·6	14·3	19·8	34·1	41·9
	Dec.	14·8	12·0	18·4	30·4	39·5
	Mean*	—	12·6	21·3	33·9	37·1
1948	Jan.	12·8	9·9	18·5	28·4	34·9
	Feb.	9·9	9·6	18·7	28·3	33·8
	Mar.	7·4	9·5	19·7	29·2	32·4
	Apr.	5·0	10·8	23·0	33·8	32·0
	May	2·5	12·2	28·0	40·2	30·3
	June	0·9	14·4	29·8	44·2	32·6
	July	1·7	17·2	30·1	47·3	36·4
	Aug.	6·0	16·0	31·3	47·3	33·8
	Sept.	9·9	15·7	26·8	42·5	36·9
	Oct.	13·8	15·5	23·9	39·4	39·4
	Nov.	15·0	14·8	21·0	35·8	41·4
	Dec.	15·2	12·8	18·7	31·5	40·7
	Mean*	—	13·0	22·2	35·2	37·0
1946–8	Mean†	—	12·9	22·3	35·2	36·8

* Weighted for differences in monthly production.
† Weighted for differences in annual production.

Table 9. *Mean annual vitamin A potency values of New Zelaand butter during* 1946, 1947 *and* 1948, *and during* 1946–8

Period	Vitamin A potency (uncorr.)		Vitamin A potency (corr.)	
	(i.u./g.)	(i.u./lb.)	(i.u./g.)	(i.u./lb.)
1946	36·8	16,690	38·6	17,510
1947	33·9	15,380	35·5	16,100
1948	35·2	15,970	36·9	16,740
1946–8	35·2	15,970	36·9	16,740

7% loss of vitamin A in the estimation. The mean annual potency of New Zealand butter *calculated on the basis used for the United States results*, would be 41·6 i.u./g. or 18,890 i.u./lb. as compared with the quoted mean value for all United States butters, 34·6 i.u./g. or 15,680 i.u./lb. This figure is of the same order as the quoted average potency (*calculated, it should be emphasized, on a basis now regarded as erroneous*) for United States summer butter, viz. 40·0 i.u./g. or 18,160 i.u./lb.

It is clear from the above comparisons that the vitamin A potency of New Zealand summer butter is as high as that of summer butter in the Northern Hemisphere countries for which results are quoted, and that the corresponding average values for New Zealand winter butters are higher than the average values for either winter or summer butters in all these countries.

Table 10. *Comparative vitamin A potencies of butter*

	Vitamin A potency expressed as			
	(i.u./g.)		(i.u./lb.)	
Country	Winter butter	Summer butter	Winter butter	Summer butter
New Zealand	41·9	33·8	19,010	15,330
Great Britain*	18·5	29·0	8,390	13,150
Sweden (29)	15·0	24·2	6,800	10,980
Denmark (9)	16·5	33·1	7,480	15,010

* Results calculated from data published by Thompson, Ganguly, Mawson & Kon (36) for carotene and vitamin A contents of milk in Great Britain. The assumption has been made that 80% of the total carotenoids in the summer milks and 75% of the total in the winter milks examined was active β-carotene.

(*e*) *Proportion of vitamin A potency due to carotene. Effect of breed of cow*

The proportion of the vitamin A potency of butterfat contributed by carotene is known to vary according to the breed of cow from which the butterfat is drawn. It is characteristic of the Jersey cow that in comparison with cows of other breeds, especially the Holstein cow, she is an inefficient converter of diet carotene to vitamin A. United States workers, for example, studying butterfats from a Jersey and a Holstein herd of cows on summer pasture, found that carotene contributed 40·3%* of the total potency of the Jersey butter and 20·9% of the potency of the Holstein butter (compare also results for Kansas butter (27) and for Swedish butter (39)). Barnicoat, working in New Zealand, obtained similar comparative results (2). Since approximately 80% of the milking cows in New Zealand are of the Jersey breed it can be expected that carotene will contribute a relatively high proportion of the total potency of New Zealand butterfat. This expectation is confirmed by the monthly figures given in Table 8 and in Fig. 4. The proportional contribution of carotene to the total potency varied from 26 to 42%, average 37%. It was greatest in the spring and early summer butterfats (average for September–December inclusive, over the three years, 40%), and lowest in the late summer/early autumn, butterfats (average, February to May inclusive, 29%). These figures may be compared with those for Minnesota butterfats, viz. 14%* when the cows were on indoor feeding and 29%* after the cows had been on pasture for some weeks (22). Carotene thus contributes a higher proportion of the total potency in New Zealand butters than in most butters reported upon elsewhere. Australian results (8) are similar to those reported here for New Zealand butterfat.

* Figures recalculated on conversion factor of 3·3 i.u./μg. of vitamin A.

The lines in Fig. 3 show that the proportional contribution of carotene to the potency in South Island butterfat is smaller than in North Island butterfat. Part of this difference is attributable to the greater proportion of cows of breeds other than Jersey in the South Island dairying districts, and probably part of it to differences in the feeding conditions of the cows in the two islands during the winter (see later).

(5) *Effect of storage on carotene and vitamin A contents of butter*

In butters reasonably free from copper and iron contamination carotene and vitamin A have been reported to be relatively stable, although, according to Garrett & Bosshardt (11), not completely stable. American workers, for example, concluded that changes in potency of American butters under normal storage were very slight (5). Butters with appreciable contents of copper, however, suffer loss in carotene and vitamin A contents through oxidation during storage.

In the present investigation all the 2-monthly samples of butter placed in storage were analysed for copper and iron contents, by the method of McDowell (16). The results are summarized in Table 11.

Table 11. *Classification of experimental storage butters according to their copper and iron contents*

Range of copper content (p.p.m.)	No. of butters	Percentage of total no.	Range of iron content (p.p.m.)	No. of butters	Percentage of total no.
0–0·19	293	87·5	0–1·99	320	95·5
0·20–0·49	37	11·0	2·00–4·50	15	4·5
0·50–1·38	5	1·5			

Table 12. *Effect of storage on mean carotene and vitamin A contents of butterfats from butters of low copper and iron contents*

Season when butter manufactured	No. of samples	Carotene in butter (μg./g.)			Vitamin A in butter (μg./g.)		
		Fresh	After 4 months	After 9 months	Fresh	After 4 months	After 9 months
Spring (Sept.–Nov.)	86	11·35	11·55	11·06	9·22	9·09	9·10
Summer (Dec.–Feb.)	68	7·86	7·64	7·35	7·17	7·10	7·05
Autumn (Mar.–May)	73	8·17	7·82	8·13	9·29	8·81	8·66
Winter (June–Aug.)	59	11·31	11·34	11·61	10·97	10·82	10·94
All seasons	286	9·70	9·62	9·54	9·11	8·90	8·88

(*a*) *Butters of low copper and iron content*

Of the 335 stored butters examined there were 286, or 85·4%, with both low copper and low iron contents (i.e. under 0·2 and 2·0 p.p.m., respectively). The average carotene and vitamin A contents of these butters on receipt and after storage for 4 and 8 months are given in Table 12, grouped according to season of production. There was an apparent increase* in the carotene content of the spring butters after 4 months' storage and of the winter butters after 9 months' storage, and there were appreciable and statistically significant decreases in the carotene content of spring butters after 9 months' storage and of the summer butters after 4 and 9 months' storage. With the exception of the autumn samples there were no statistically significant losses in vitamin A content of the butters after 4 and 9 months' storage.

* Similar unexplained increases in carotene content have been noted in North Carolina and California (5).

(*b*) *Butters of high copper or iron content*

The recorded changes in the carotene and vitamin A contents of the thirty-seven butters with high and medium copper contents and of the fifteen butters with high iron contents, shown in Table 13, were not statistically significant.

(*c*) *Summary of results of storage trials*

The samples used in the survey were commercial butters from a representative section of New Zealand butter factories, the period of storage was greater than is normal for New Zealand butter exported overseas, and the temperatures to which the butters were subjected between time of manufacture and time of placing in cold storage were at times much higher than obtains under commercial conditions of handling of butter from the factory to the export cold-store. The indications are, therefore, that New Zealand export butter reaches the consumer in the country of its destination with its vitamin A potency unimpaired. This conclusion is in keeping with the earlier finding of Crawford *et al.* (7), who, by rat-feeding trials, did not find any significant losses in vitamin A potency of New Zealand butter stored for one and two years at 9–13° F.

Table 13. *Effect of storage on the mean carotene and vitamin A contents of butterfat from butters of appreciable copper and iron contents*

		Carotene in butter (μg./g.)			Vitamin A in butter (μg./g.)		
Metallic contamination in butter	No. of samples	Fresh	After 4 months	After 9 months	Fresh	After 4 months	After 9 months
High copper, 0·5–1·38 p.p.m.	5	10·40	10·08	10·22	10·66	10·36	10·40
Medium copper, 0·2–0·49 p.p.m.	32	9·24	9·19	9·20	9·44	9·20	9·06
High iron, 2·00–4·50 p.p.m.	13	9·33	9·28	9·47	9·58	9·48	9·51

DISCUSSION

The variations in carotene content, vitamin A content, and vitamin A potency of New Zealand butterfat, as reported above, are of particular interest (i) because of the regularity of the seasonal changes in these values for the butterfats from the four North Island districts, (ii) because of the consistent differences between the North Island and the South Island results, and (iii) because of the difference in the trend of the seasonal changes in comparison with that of the seasonal changes in northern hemisphere countries. The maximum values in New Zealand, for example, are found in the winter butterfats, whereas in northern hemisphere butterfats the maximum values are found in the summer butterfats. Results recently reported for Victorian butterfats (8) are similar to those recorded in the present paper (and to those for butterfats analysed in Montana, U.S.A. (5)). It is of interest to consider the possible causes of these seasonal changes.

(*a*) *Carotene and vitamin A contents of butterfat in relation to period of lactation of cows*

Dairying for manufacturing purposes in New Zealand is highly seasonal. The cows are calved down during the early spring (July, August and September) and are dried off in the late-autumn/early-winter period. As a result the maximum production of butter is obtained in the late-spring/early-summer period (see Table 8). Any general lactational change in the carotene or vitamin A contents of butterfat, therefore, could be expected to show up as a change in the carotene and vitamin A contents of commercial New Zealand butter.

Butterfat from milk yielded by cows immediately after parturition—the colostral fat—is known to have a higher carotene and vitamin A content than that from milk produced later in the lactation period. The contents are highest in the early post-parturition fat, and there is usually a rapid decline to more or less normal values by the third day after parturition. If, however, the cow has had access to pasture during the pre-partum period, high carotene and vitamin A contents may persist for 4–8 days after parturition (13, 28, 35). Thus Barnicoat (2) and McGillivray (18) have shown that under North Island spring conditions of grazing the carotene and vitamin A contents of Jersey butterfat produced by cows at 6–8 days after calving are higher than in the later, more normal, samples. Walker, Thompson, Bartlett & Kon (37), on the other hand, found that even with a high level of carotene feeding for 90 days before calving (the carotene was administered as a solution in oil) the carotene content of the butterfat reached a normal level within 7 days of calving; and with a high level of vitamin A feeding before calving the vitamin A content of the butterfat became normal within 5 days. Apart from these high values in the early post-colostral fat there is no reported occurrence* in overseas countries of any purely lactational variations in the carotene and vitamin A contents of butterfat. Hibbs, Krauss & Monroe (14), for example, state that 'a study of their milk production curves revealed no indication that the milk carotenoids were influenced markedly by the stage of lactation', and Barnicoat (4) found that the carotene and vitamin A contents of butterfats drawn at the same time from autumn-calving and spring-calving cows kept under identical feeding conditions were of the same order of magnitude, despite the 5 months' interval between the dates of commencement of lactation of the two groups.

The extent of the possible influence of the high vitamin A potency of post-colostral butterfat on the vitamin A potency of butterfats from New Zealand factory-made butters depends on a number of factors.

(i) *Period allowed after the calving before the milk is taken for separation of cream for butter-making*

The usual requirement is that milk produced during the first 4 days of the lactation period shall be discarded. Where this requirement is followed the main potential effect of the high vitamin A potency of post-colostral fat on the potency of butterfat from commercial butter will be eliminated as a factor of major importance, but there still could be some effect because higher-than-normal potency values may persist for several days after this 4-day post-colostral period (3, 28).

(ii) *Relationship of amount of post-colostral fat to total amount of fat in the butter*

This will be affected by the number of stripper cows carried over from the previous season, the rate at which cows come into profit during the spring months, and the effect of feeding conditions on volume of butter production. The figures in Table 14 show that a large proportion of cows in New Zealand are brought to profit during July and August, the period when contents of carotene and vitamin A in the butterfat are at their maximum. When account is taken of the possible proportion of early post-parturition fat in the butter, however, it is obvious that the high carotenoid contents of New Zealand butterfat in the early spring months cannot be due to inclusion of post-colostral fat—the values

* Winzenried and Wanntorp (39) from results obtained with identical twin cows have reported a falling off in potency towards the end of lactation.

should fall as the proportion of the number of newly calved cows to total number of cows decreases during July and August, whereas reference to Table 2 and Figs. 1A and 1B shows that, on the contrary, the values continue to rise during August. Furthermore, it is clear from a study of the lines in Figs. 1, 2A, 2B, 3 and 4: (*a*) that the rises in carotene and vitamin A contents commence in the late autumn and continue throughout the winter period, and that, therefore, the high values for July and August butterfats are the continuation of a trend already in progress before the spring, i.e. the calving season; and (*b*) that in some years the high carotene values persist for such a long period that they could not possibly be attributed to the presence of post-colostral fat in the butter (e.g. in the South Island the maximum persists until November). It may be concluded, therefore, that the peculiar seasonal changes in vitamin A potency of New Zealand butterfat are not caused solely by purely lactational influences.

Table 14. *Percentages of cows tested by Dominion Herd Improvement Association calving during the early spring months*

Period	Percentage of yearly number of cows		Cumulative percentage
June	2·84		0
1–15 July	6·93	25·84	28·68
16–31 July	18·91		
1–15 August	21·96	38·92	67·60
16–31 August	16·96		

(iii) *Relation of feeding conditions to carotene and vitamin A contents of butterfat*

With cows under stall-feeding conditions the variations in carotene and vitamin A contents of the butterfats reflect the variations in the carotene and vitamin A contents of the feeds consumed by the cows. Under pasture-feeding conditions, however, the relationship is somewhat different, because the carotene content of the feed is then frequently in excess of requirements. Mitchell & Wise (21), for example, from their investigations on the carotene content of pasture in relation to that of the milk produced therefrom, concluded that 'it is reasonable to assume that any pasture providing the quality and the quantity of herbage necessary to maintain optimum milk yields over a prolonged period also would supply carotene in sufficient quantity to raise the concentration in the milk to "ceiling value"'. Hibbs *et al.* (14), in Ohio, also working with Jersey cows, found that after the initial rise following change from stall to pasture-feeding the milk carotenoid content did not follow closely the changes in pasture carotenoid content except when the pasture carotenoid level was below 26 mg./100 g.* They suggested that there was a maximum response at this level. They found, too, that the vitamin A content of the milk did not respond to changes in the carotenoid content of the herbage as readily as did the carotene content. Some workers (1,3,21,38) have stated that the daily intake of carotene necessary to maintain the vitamin A potency of butterfat at a maximum level ranges from 770,000 to 1,500,000 i.u. per day, but Wiseman, Sheppard & Cary (40) concluded that an intake of approximately 2500 mg. (4,175,000 i.u.) of carotene is required to maintain maximum vitamin A potency. Intake of carotene above a certain level was reported also by Sen & Ray Sarkar (31,32) to have no beneficial effect on the potency of the butterfat.

* McGillivray (19) has found that the carotenoid contents of New Zealand pasture may fall during drought periods to as low as 15 mg./100 g.

The feeding conditions for cows in New Zealand, especially for those in the North Island, differ considerably from those in most northern hemisphere countries, in that the cows are at pasture throughout the year, and that even during winter in the main butter-making districts of the North Island pasture forms an appreciable portion of the ration. When supplementary feeds are given, as in periods of dry weather in the late summer and early autumn, and during winter, they consist usually of carotene-containing products, such as grass and clover silage, hay, choumoellier and green maize—there is, even in the winter, very little of the feeding of grains and other similar low-carotene-content feeds so regularly adopted in northern hemisphere countries.

The percentage carotene content of North Island pastures is reported (25,26,33) to be fairly uniform throughout the spring and early summer, within the range 40–50 mg./100 g. of moisture-free material. There is no evidence of a general fall in carotene content of pasture from September to October onwards, which might account for the constant decline in carotene and vitamin A contents of the butterfat during this period, reported both in the present investigation and by Barnicoat (2) for New Zealand, and by Farrer, Balding, Warren & Millar (8) for Australia; and, furthermore, the amount of carotene in the pasture is considerably higher than that found by Hibbs *et al.* (see above) to give maximum response under Ohio conditions. If the carotene and vitamin A contents of North Island butterfat were directly related to the percentage carotene content of the feed, it would be expected that they would tend to rise, rather than fall, during the late spring and early summer months; because at this period there is usually a prolific growth of ryegrass and clover of high carotene content, with the result that the cows are ingesting 5–10 times the amount of carotene found by Wiseman (see above) to be necessary to maintain vitamin A potency at a maximum. The decline in carotene and vitamin A contents, and therefore in vitamin A potency, in the late-spring/early-summer period, therefore, must be attributed not to lack of carotene in the diet, but, at least in part, to a poor rate of utilization of the carotene in the grass by the cow during this period. It is well known that there are wide variations in the availability of carotene from different sources (12), possibly due to variation in the amount of tocopherol or other antioxidant in the pasture (19). The major fall in vitamin A potency of the butterfat in New Zealand occurs at approximately the same time as the emergence of clover as the dominant constituent in the pasture, but at time of writing there is no experimental evidence showing a connexion between this change in the pasture type and the change in carotene and vitamin A contents of the butterfat.

(iv) *Relation of season and weather conditions to carotene and vitamin A contents of butterfat*

Season and weather conditions could conceivably influence carotene and vitamin A contents of butterfat:

(*a*) Directly, by their effect on the metabolism of the cow. On this aspect there is little published information but Stallcup & Herman (34) have recently concluded that 'environmental temperature *per se* does not appear to markedly influence the carotenoid and vitamin A levels in milk fat and blood plasma—the variations in values observed were closely associated with dietary intake which is influenced by environmental temperature'.

(*b*) Indirectly, for cows at pasture, by their effect on the character and composition of the plants in the pasture. It could be expected, therefore, that butterfat produced during periods of drought, i.e. when the pastures are dried up and brown and therefore of low

carotene content, would be of low carotene and vitamin A contents. For butterfat, produced under farming conditions in New Zealand, however, an exact relationship between weather conditions and carotene and vitamin A contents of butterfat is not to be anticipated, for the reason that the supplementary feeds given to the cows in periods of pasture scarcity are mainly grass silage, hay, green maize, choumoellier and similar products, all of them carotene-containing. The extent to which a period of drought will affect the carotene intake of cows in New Zealand will therefore vary according to whether the previous seasonal conditions permitted the accumulation of an adequate stock of these supplementary feeds in good condition, and according to the length of the drought period in relation to the supply of supplementary feeds on hand.

Because of the above confusing factors the results obtained in the survey under review cannot serve as a basis for study of the effect of drought, *per se*, on the carotene and vitamin A contents of butterfat, but they are of value for the study of the effect, if any, of weather conditions on New Zealand commercial butterfat. Over the 3½ years during which the analyses were made there were severe droughts in districts 1, 2 and 3 in the summer and autumn of the 1945–6 season, and in district 4 in the summer and autumn of the 1947–8 season. The lines in the left-hand graph of Fig. 5 show the average carotene and the vitamin A contents of butterfat obtained from a factory in district 2, plotted at fortnightly intervals over the whole period of the survey but with the graphs for the three years superimposed. At this factory (factory I) the fall in carotene content in the drought year, 1945–6, was not significantly greater than in the two subsequent years when there were no drought conditions. There was a sharp rise in vitamin A content of the butterfat during this period, and, therefore, a distinctly higher vitamin A potency. The results for factory I are typical of those for all factories in districts 1, 2 and 3; but a similar rise in vitamin A content did not occur in the butterfat from the factories in district 4 for this same year. There is thus what may be called 'circumstantial evidence' that the drought caused the peculiar rise in vitamin A content of butterfat in districts 1, 2 and 3 in 1945–6. At factory Q (in district 4) during the drought period of 1948 (November to March), on the other hand, the carotene and vitamin A contents of the butterfats were lower than during the more normal seasons of 1946–7 and 1947–8 (see Fig. 5). It is possible that drought in subdistrict Q would have a different effect on feeding conditions than in subdistrict I, owing to differences in soil conditions or type of pasture. Whatever the cause of the variations in the effect of drought as between butterfats from factory I and factory Q, the results presented suffice to establish that the system of maintaining cows under New Zealand dairy-farming conditions provides a reasonably satisfactory buffering effect against changes in carotene and vitamin A content of butterfat which otherwise might result from the effect of drought conditions on pasture growth, and that the average values quoted in the present paper are likely to represent a significant average for New Zealand butterfat in any dairying year.

(v) *Carotene and vitamin A contents of butterfat in relation to milk and butterfat yield*

The late-spring/early-summer fall in carotene and vitamin A contents of New Zealand butterfat occurs simultaneously with the rise to maximum yield of butterfat. Prior to this rise in yield the cows are somewhat underfed, because of the lack of adequate pasture, and are drawing to some extent on their body reserves to maintain their milk supply. As a consequence of the feeding conditions in New Zealand the lactational curve of milk

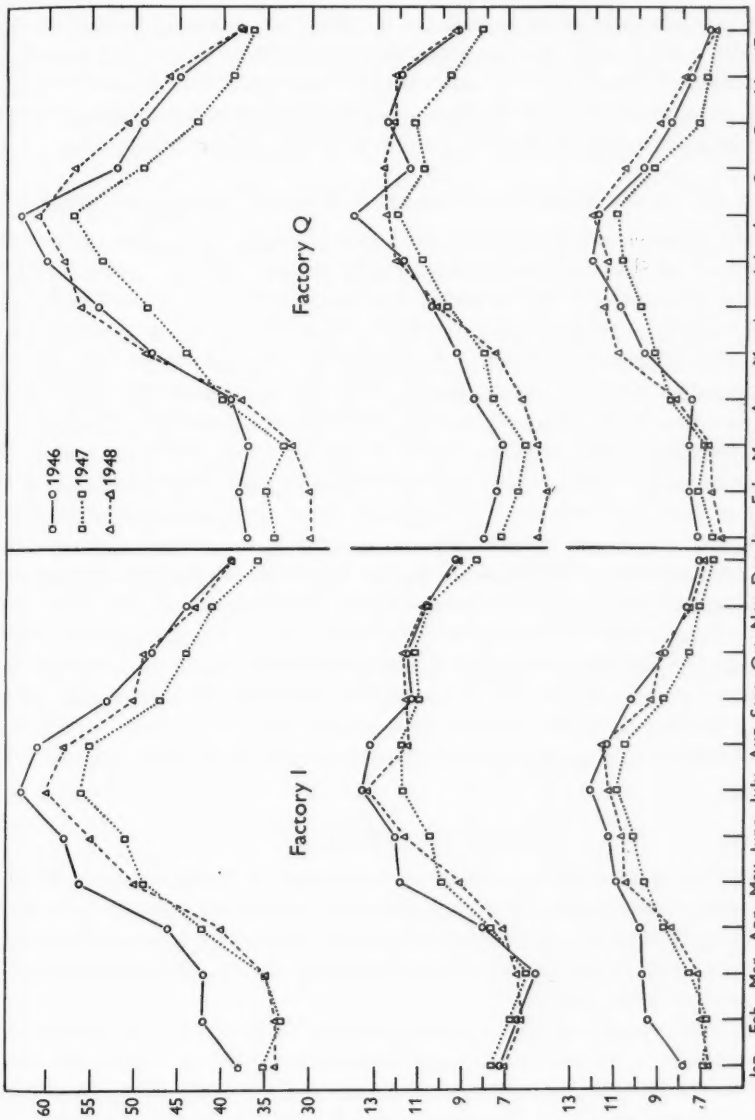
Factory Q
Factory I
1946
1947
1948
Total vitamin A potency (i.u./g.)
Carotene (μg./g.)
Vitamin A (μg./g.)
Jan. Feb. Mar. Apr. May June July Aug. Sept. Oct. Nov. Dec. Jan. Feb. Mar. Apr. May June July Aug. Sept. Oct. Nov. Dec.
Month of year

Fig. 5. Mean monthly values for carotene and vitamin A contents and for vitamin A potency of butterfats from factory I (district 2) and factory Q (district 4) superimposed for the three years of the survey.

production is abnormal, in that the maximum yield is reached at a later stage than in northern hemisphere countries where cows are fed by rationing to give the maximum yield as soon as possible after calving. The rise to high carotene and vitamin A contents in the butterfat in New Zealand during the autumn and winter months occurs simultaneously with the lactational fall in milk and fat yield; and during periods of drought there is usually a fall in milk and fat yield. There is at present no evidence to connect the changes reported here in carotene and vitamin A contents of New Zealand butterfat with the rise in milk and fat yield during the late-spring/early-summer period, or with the fall in milk and fat yield during the autumn or during a period of drought.

(vi) *Carotene and vitamin A contents of butterfats in relation to butterfat characteristics*

In northern hemisphere countries the *sharp rise in vitamin A potency* of butterfat following transfer of the cows from stall-feeding to pasture-feeding is accompanied by a *sharp rise in iodine value* of the butterfat. The rise in vitamin A potency of Swedish butterfat, for example, from 18 i.u./g. in March–April–May to 29 i.u./g. in June and 34 i.u./g. in July (reported by Platon & Swartling(29)), runs roughly parallel with the regular rise in iodine value from approximately 30 in May to 42–44 in June (reported by Platon & Olsson(30)). In New Zealand, the sharp fall in vitamin A potency of the butterfat in the late-spring/early-summer period occurs at approximately the same time as the sharp decline in iodine value of the butterfat recorded by Cox & McDowall(6), and the autumn rise in vitamin A potency is accompanied by a corresponding seasonal rise in iodine value. The parallelism between the seasonal trends in carotene and vitamin A contents and iodine values is illustrated in Fig. 3. It will be seen that the divergence in the carotene and vitamin figures as between North and South Island butterfats corresponds approximately with the divergence in the iodine values. These similarities suggest that the changes in vitamin content and in butterfat composition are controlled by the same causative factor—perhaps the change in the tocopherol content of the pasture suggested by McGillivray as the possible cause of the changes in vitamin A potency in New Zealand butterfat, or, perhaps, variations in the nature of the fatty materials in the pasture.

SUMMARY AND CONCLUSIONS

Results of a survey of the carotene and vitamin A contents, and of the vitamin A potency, of New Zealand butterfat and butter, are presented. The butter samples were drawn from twenty representative commercial factories at fortnightly intervals over three successive years (1946–8). The survey covers the analysis of 1517 samples of fresh butter and 670 samples of stored butter.

There were uniform and regularly recurring seasonal variations in both carotene and vitamin A contents of butterfats from all North Island districts. Maximum values were found in the late-autumn/winter/early-spring, and minimum values in the late-summer/early-autumn butterfats. The seasonal trends were thus distinctly different from those which have been reported for northern hemisphere butterfats, for which the maximum values are commonly found during the summer grazing period.

The seasonal variations in carotene and vitamin A contents of South Island butterfat were less marked and less consistent than those in the corresponding values for North Island butterfat.

It is of interest that the seasonal changes in carotene and vitamin A contents run parallel with the seasonal changes in iodine values of the butterfats, both in New Zealand and in northern hemisphere countries.

Drought conditions accompanied by drying-up of pastures did not lead to the expected fall in carotene and vitamin A contents of the butterfat, probably because of the use of silage and other green plant material as supplementary feeds during the dry-weather period.

The contribution of carotene to the total vitamin A potency of New Zealand butter varied from 26 to 42%, average 37%. This high proportional contribution of carotene to the total potency is due to the preponderance of cows of the Jersey breed in New Zealand dairy herds.

The mean annual carotene and vitamin A contents of North Island butterfat were 9·54 and 8·12 μg./g., and those of South Island butterfat were 8·41 and 8·57 μg./g., respectively.

The highest vitamin A potency values (45·0–50·2 i.u./g.) were found in the winter (July–August) butter, and the lowest potency values (28·3–33·4 i.u./g.) in the summer/early-autumn (January, February, March) butter. The average vitamin A potency found for New Zealand winter butter (April to August inclusive, representing 17% of the annual production) was 41·9 i.u./g. or 19,010 i.u./lb. The average for New Zealand summer butter (September to March inclusive, representing 83% of the annual production) was 33·8 i.u./g., or 15,330 i.u./lb.

The mean vitamin A potency (uncorrected for loss of vitamin A during analysis) of New Zealand butter over the three years was 35·2 i.u./g. or 15,970 i.u./lb.; and with application of a 7% correction for loss of vitamin A in analysis it would be 36·9 i.u./g. or 16,740 i.u./lb.

There was no significant loss in vitamin A potency of New Zealand commercial butter during storage for 8 months at 14° F.

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REFERENCES

(1) Anon (1944). *Nutrit. Rev.* **2**, 203.
(2) Barnicoat, C. R. (1947). *J. Dairy Res.* **15**, 80.
(3) Baumann, G. A. & Steenbock, H. (1934). *J. biol. Chem.* **105**, 167.
(4) Berl, S. & Peterson, W. H. (1943). *J. Nutrit.* **26**, 527.
(5) Cary, C. A. and others (1947). *Miss. Publ. U.S. Dep. Agric.* no 636.
(6) Cox, G. A. & McDowall, F. H. (1948). *J. Dairy Res.* **15**, 377.
(7) Crawford, M. F. F., Perry, V. O. U. & Zilva, S. S. (1932). *Spec. Rep. Ser. med. Res. Coun., Lond.*, no. 175.

(8) Farrer, K. T. H., Balding, W. M., Warren, H. S. & Millar, R. G. (1949). *Aust. J. Sci. Res.* B, **2**, 355.
(9) Fridericia, L. S. (1947). *Proc. Nutr. Soc.* **5**, 255.
(10) Frengley, M. G. & Herrick, M. M. (1931). *Bull. N.Z. Dep. sci. industr. Res.* no. 34.
(11) Garrett, O. F. & Bosshardt, D. K. (1944). *N.J. agric. Exp. Sta.* no. 710.
(12) Guggenheim, K. (1944). *Biochem. J.* **138**, 260.
(13) Henry, K. M., Houston, J. & Kon, S. K. (1940). *J. Dairy Res.* **11**, 1.
(14) Hibbs, J. W., Krauss, W. E. & Monroe, C. F. (1949). *J. Dairy Sci.* **32**, 955.
(15) Jenness, R. & Palmer, L. S. (1945). *J. Dairy Sci.* **28**, 473.
(16) McDowell, A. K. R. (1947). *J. Dairy Res.* **15**, 70.
(17) McDowell, A. K. R. (1949). *J. Dairy Res.* **16**, 348.
(18) McGillivray, W. A. (1949). Massey Agricultural College, Palmerston North, N.Z. Private communication.
(19) McGillivray, W. A. (1952). *J. Dairy Res.* **19**, 119.
(20) Malcolm, J. & Pope, E. A. (1929). *N.Z. J. Sci. Tech.* **11**, 228.
(21) Mitchell, J. H. & Wise, G. H. (1944). *J. Dairy Sci.* **27**, 189.
(22) Morgan, R. S. & Pritchard, H. (1937). *Analyst*, **62**, 354.
(23) Morton, R. A. (1945). Private communication.
(24) New Zealand Dairy Board (1947, 1948). *Annual Reports.*
(25) New Zealand Dep. Agric. Animal Res. Sta. Ruakura (1949). Unpublished observations.
(26) New Zealand Dep. Sci. Industr. Res. Plant Chemistry Laboratory, Palmerston North (1949). Unpublished observations.
(27) Parrish, D. B., Martin, W. H., Atkeson, F. W. & Hughes, J. S. (1946). *J. Dairy Sci.* **29**, 91.
(28) Parrish, D. B., Wise, G. H., Atkeson, F. W. & Hughes, J. S. (1949). *J. Dairy Sci.* **32**, 209.
(29) Platon, B. & Swartling, P. (1944). *Medd. Statens Mejeriförsök, Alnarp*, no. 12.
(30) Platon, B. & Olsson, T. (1941). *Medd. Statens Mejeriförsök, Alnarp*, no. 8.
(31) Ray Sarkar, B. C. & Sen, K. C. (1943). *Indian J. vet. Sci.* **13**, 219.
(32) Sen, K. C. & Ray Sarkar, B. C. (1942). *Indian J. vet. Sci.* **12**, 179.
(33) Shorland, F. B. (1949). *J. N.Z. Inst. Chem.* **13**, 13.
(34) Stallcup, O. T. & Herman, H. A. (1950). *Res. Bull. Mo. Agric. Exp. Sta.* no. 457.
(35) Sutton, W. S., Warner, R. G. & Kaeser, H. E. (1947). *J. Dairy Sci.* **30**, 927.
(36) Thompson, S. Y., Ganguly, J., Mawson, E. H. & Kon, S. K. (1949). *Rep. 12th Int. Dairy Cong., Stockholm*, **2**, 238.
(37) Walker, D. M., Thompson, S. Y., Bartlett, S. & Kon, S. K. (1949). *Rep. 12th Int. Dairy Congr., Stockholm*, **1**, 83.
(38) Wilbur, J. W., Hilton, J. H. & Hauge, S. M. (1940). *J. Dairy Sci.* **23**, 765.
(39) Winzenried, H. U. & Wanntorp, H. (1948). *Int. Z. Vitaminforsch.* **20**, 134.
(40) Wiseman, H. G., Sheppard, J. B. & Cary, C. A. (1949). *Rep. 12th Int. Dairy Congr., Stockholm*, **1**, 61.
(41) World Health Organization (1950). *Tech. Rep. Series*, 3 (Brit. Abstr. (1950) A III, 1634).
(42) Zscheile, F. P., Henry, R. L., White, J. W., Nash, H. A., Shrewsbury, C. L. & Hauge, S. M. (1944). *Industr. Engng Chem.* (Anal. ed.), **16**, 190.

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503. THE PROPERTIES OF NEW ZEALAND BUTTERS AND BUTTERFATS

III.* SEASONAL VARIATIONS IN THE NATURE OF THE UNSATURATED ACIDS OF BUTTERFAT AS ESTIMATED BY SPECTROPHOTOMETRIC METHODS

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(With 1 Figure)

A consistent seasonal variation in the iodine values of butterfats from all districts of the North Island of New Zealand was reported by Cox & McDowall (1). The relation of the seasonal change in iodine value to the change in unsaturated fatty acid composition of the butterfat as determined by the ester fractionation method has been examined by Hansen & Shorland (2). The present paper covers the results of a study of the relationship between iodine value and type of unsaturated acid—monoenoic, dienoic, trienoic, etc.—estimated by the absorption spectrophotometric method.

Gillam, Heilbron, Hilditch & Morton (3), in 1931, showed that the fatty acids obtained by saponification of butterfat at 150–160° for 2 hr. were characterized in alcoholic solution by a number of absorption bands in the region 220–320 mμ. Dann & Moore (4) suggested that these absorption bands which appeared in the fatty acids obtained by prolonged saponification of butterfat, and also of many other fats and oils, were due to isomeric changes in the highly unsaturated acids. Booth, Kon, Dann & Moore (5) found that the acids from butterfat obtained by saponification for only 2 min. gave a definite absorption at 230 mμ, whereas acids obtained from most other fats and oils by a similar treatment showed no absorption. (Booth *et al.* found also that there were seasonal variations in the spectroscopic readings at 230 mμ for butterfat acids obtained after a short saponification.) This peculiar absorptive property of butterfat was attributed by later workers to a conversion in the cow's body of 'pro-absorptive' acids (i.e. acids developing only after prolonged saponification) to 'absorptive' acids (i.e. acids developing absorptive properties after a short period of saponification). Dann, Moore, Booth, Golding & Kon (6), for example, found that cod-liver oil, sardine oil, rape oil and linseed oil, none of which gave acids showing absorption until after prolonged saponification, when added to the cow's ration caused a considerable increase in the proportion of acids in the butterfat showing absorption at 230 mμ. The unsaturated acids in dried grass fat† were found by Houston, Cotton, Kon & Moore (7) and by Moore (8) to be in the 'pro-absorptive' state, and the relatively high content of 'absorptive' acids in butterfat

* For parts I and II of this series see Cox & McDowall (1) and Dolby, R. M. (1949), *J. Dairy Res.* **16**, 336.

† Hilditch & Jasperson (9) found in grass fats about 3% of conjugated *diene* C_{18} acids and a very small proportion of conjugated *triene* C_{18} acids.

from cows on pasture was attributed by these authors to a conversion of the 'pro-absorptive' grass fat acids in the animal's body. Houston *et al.* (7) also confirmed the principle that spectroscopic absorption, whether actual or potential in butterfat, is concentrated in the more highly unsaturated acids of the fat; and Moore (8), in 1939, showed that the increase in absorption occurring in polyethylenic acids during prolonged saponification is caused by a conversion of non-conjugated ('pro-absorptive') unsaturated linkages to conjugated ('absorptive') linkages.

Use of the absorption spectrophotometer for quantitative estimation of the degree of unsaturation in the acids of fats and oils was described by Mitchell, Kraybill & Zscheile (10), and later, in more detail, by Brice & Swain (11, 12). The content of conjugated acids was estimated by spectral density readings at certain wave-lengths on a solution of the fat in an organic solvent, and the content of non-conjugated acids by further readings on an alcoholic solution of the alkali-isomerized fat. Baldwin & Daubert (13) and Mattson (14) found that unsaturated acids in the form of synthetic glycerides (13) or in butterfat (14) gave substantially the same absorption results as the acids obtained after a short saponification.

EXPERIMENTAL

Sampling and preparation of butterfat

The butterfats examined were all obtained from butter churned at a large butter factory in the Manawatu district of the North Island, over the period October 1949 to April 1950. In view of the consistency of the seasonal variations noted by Cox & McDowall (1), the butterfats can be taken as fairly representative of North Island butterfat. The butterfat samples were prepared by melting the fresh butter, decanting the butterfat, and filtering at 60°.

Measurement of optical density

The instrument used was a Beckman D.U. photoelectric spectrophotometer with hydrogen-lamp attachment.

Estimation of unsaturated acid constituents

A comparison was made of the absorption in untreated butterfat with absorption in the acids extracted after short saponification. The results confirmed those of Baldwin & Daubert and of Mattsson, referred to above, that the saponification made little difference to the result. The measurements, therefore, were made on the butterfat itself.

(*a*) *Conjugated acids.* Melted butterfat (0·1 g.) was dissolved in 200 ml. light petroleum* (b.p. 55–65°). Density measurements were made at 232 mμ (dienoic acids), 262, 268 and 274 mμ (trienoic acids) and 310, 316 and 322 mμ (tetraenoic acids). The results were converted to percentage values for each type of acid, according to the methods of calculation described by Brice & Swain (11), i.e. correcting for the slight absorption due to the COOH radicle and the multiple C=C groups at 232 mμ (absorption maximum for dienoic acids) and also for 'background absorption' due to less saturated conjugated acids and to unknown constituents at 268 and 316 mμ (the absorption maxima for trienoic and tetraenoic acids respectively).

* Almost identical values for specific absorption were obtained using absolute alcohol (14), cyclohexane (12), or light petroleum as solvents.

(*b*) *Non-conjugated acids.* Butterfat (0·1 g.) was isomerized in an ethylene glycol solution* of KOH, as described by Mitchell *et al.* (10). The isomerized mixture, after cooling, was transferred to a 100 ml. flask and made to the mark with absolute alcohol which had been redistilled from KOH and zinc dust. It was then left overnight and filtered. Density measurements were made on the undiluted filtrate in the range 310–322 mμ, and on a further portion of the filtrate, diluted five times with absolute alcohol, in the range 262–274 mμ and at 232 mμ. The percentage of each non-conjugated acid constituent was calculated from Brice & Swain's equations (11), which include corrections for (*a*) 'background' absorption, (*b*) the lower unsaturated acids which result from the alkali-isomerization of non-conjugated tetraenoic and trienoic acids, and (*c*) the specific absorption values of the conjugated acids which, as these and other workers have shown (11, 14), are not affected by alkali isomerization. This method was found to give results for content of non-conjugated dienoic and trienoic acids similar to those obtained by the isomerization technique of Hilditch, Moore & Riley (16) (estimation of dienoic and of trienoic acids on separate portions of the same sample). It was preferred to Hilditch's method, however, because the estimation of all polyethylenic non-conjugated acids could be carried through on the same isomerized solution.

Estimation of iodine values

The iodine values of the butterfats were determined by the British Standard Method (17). Iodine values for each unsaturated acid constituent are quoted in the literature (15); and these values, together with the estimated percentage content of each acid in the butterfat, furnished sufficient data for the calculation of the oleic acid content by difference from the iodine value of the fat. The small proportions of C_{10}, C_{12}, C_{14} and C_{16} monoenoic acids found in North Island butterfat in addition to the C_{18} acid (2) also will be included in the 'oleic' acid estimated by difference.

RESULTS

The iodine values and the percentage contents of each unsaturated acid constituent of the butterfats examined are given in Table 1. The figures both for iodine value and for percentage of each unsaturated acid are reasonably close for all butterfats sampled in the same month. The average monthly results are shown in Table 2. The average monthly figures for iodine value and for content of oleic acid are plotted in Fig. 1, along with the average monthly iodine values for seven North Island factory butterfat samples from October to April over 4 years (Cox & McDowall (1)). It will be seen that the seasonal trends in iodine value of the butterfats as reported in the present investigation are very similar to those reported by Cox & McDowall.

Oleic acid

The average monthly percentage contents for oleic acid fell from 33·8 in October to 29·3 in December and rose to 35·8 in April. No comparison with results for oleic acid

* Use of this ethylene glycol solution of KOH for isomerization was criticized by Brice & Swain (11, 12) on the grounds that it developed a strong and variable ultra-violet absorption on heating in air, and that the 'background absorption' by extraneous compounds was relatively high. (This difficulty has since been overcome by nitrogen 'blanketing' during preparation of the reagent and during the isomerization process (15)). They preferred a solution of KOH in glycerol. In the present author's hands, however, this latter reagent did not prove satisfactory—isomerization was incomplete and the isomerized mixture after cooling was, because of its viscous nature, very troublesome to deal with.

content, determined by the ester fractionation method by Hansen & Shorland(2), is possible, since these authors considered that their data were insufficient to resolve C_{18} unsaturated acids into their components. The percentage contents of dienoic (conjugated + non-conjugated), trienoic and tetraenoic acids were small in comparison with those for oleic acid (Table 2), and any fluctuations from month to month were also fairly small. Seasonal variations in iodine value must, therefore, be due mainly to variations in oleic acid content (compare, for example, the relationship shown in Fig. 1). Hansen & Shorland(2), in their investigation of Rangitaiki Plains (North Island) butterfat,

Table 1. *Iodine values and percentage contents of each unsaturated constituent of the butterfats examined*

Churning date		Iodine value	Oleic acid (%)	Dienoic acids (%)		Trienoic acids (%)		Tetraenoic acids (%)	
				Conjugated	Non-conjugated	Conjugated	Non-conjugated	Conjugated	Non-conjugated
1949	14/x	36·5	33·5	1·3	0·3	0·03	1·0	Nil	0·3
	21/x	37·0	34·1	1·4	0·2	0·04	0·8	Nil	0·3
	28/x	36·7	33·7	1·4	0·3	0·03	0·8	Nil	0·3
	4/xi	34·7	31·5	1·3	0·3	0·03	0·8	Nil	0·3
	11/xi	33·6	30·4	1·2	0·4	0·03	0·8	Nil	0·4
	18/xi	33·2	30·4	1·1	0·5	0·03	0·7	Nil	0·3
	25/xi	32·3	29·6	1·0	0·4	0·04	0·7	Nil	0·3
	2/xii	31·6	28·9	0·9	0·4	0·03	0·7	Nil	0·4
	16/xii	32·5	29·6	0·9	0·5	0·03	0·8	Nil	0·3
1950	13/i	33·7	30·8	0·8	0·7	0·03	0·8	Nil	0·3
	28/i	32·2	29·4	0·8	0·5	0·03	0·9	Nil	0·3
	10/ii	32·1	30·7	0·7	0·3	0·02	0·6	Nil	0·3
	16/ii	36·3	34·8	0·9	0·2	0·03	0·7	Nil	0·3
	3/iii	34·8	32·8	0·9	0·4	0·03	0·7	Nil	0·3
	7/iii	36·9	34·0	1·2	0·9	0·03	0·5	Nil	0·4
	3/iv	37·9	35·8	1·3	0·2	0·04	0·7	Nil	0·3

Table 2. *Average monthly iodine values and average monthly percentage contents of unsaturated acid constituents of the butterfats examined*

Month		Iodine value	Oleic acid (%)	Dienoic acids (%)		Trienoic acids (%)		Tetraenoic acids (%)	
				Conjugated	Non-conjugated	Conjugated	Non-conjugated	Conjugated	Non-conjugated
1949	Oct.	36·7	33·8	1·4	0·3	Trace	0·9	Nil	0·3
	Nov.	33·5	30·5	1·2	0·4	Trace	0·8	Nil	0·3
	Dec.	32·1	29·3	0·9	0·5	Trace	0·8	Nil	0·4
1950	Jan.	33·0	30·1	0·8	0·6	Trace	0·9	Nil	0·3
	Feb.	34·2	32·7	0·8	0·3	Trace	0·7	Nil	0·3
	Mar.	35·9	33·4	1·1	0·7	Trace	0·6	Nil	0·4
	Apr.	37·9	35·8	1·3	0·2	Trace	0·7	Nil	0·3

concluded that although the iodine values were not directly proportional to the C_{18} unsaturated acid contents they were an indication of the amount of these acids present. As the seasonal variation in iodine values reported by Hansen & Shorland(2)) and by the present author follow a very similar trend to the corresponding variations for butterfats from seven North Island factories over 4 years reported by Cox & McDowall (Fig. 1), it seems reasonable to assume that in any North Island butterfat fluctuations in iodine value and in oleic acid content are closely related.

Conjugated dienoic acids

Maximum content of conjugated dienoic acids was found in October (average 1·4%) and April (1·3%), and minimum content (0·8%) in January and February (Table 2). There is thus a seasonal trend not unlike that shown by oleic acid.

Mattsson (14) reported a greater range of results for content of conjugated dienoic acids in Swedish butterfat, viz. 0·6–2·4 % (one butterfat 3·7 %), but the lower results were from the butterfats of stall-fed cows. Booth *et al.* (5) reported $E^{1\%}_{1\,cm.}$ values at 230 mμ for butterfat acids (saponification time 2 min.) for English winter and summer butterfats. Calculated as conjugated dienoic acids ($E^{1\%}_{1\,cm.}$ for conjugated dienoic acids, unisomerized = 1200 (9)) their figures are: winter butterfat 0·8 %, summer butterfat 2·1 %. Hilditch & Jasperson (18) from work on butterfat fractions found only 0·5 % of conjugated dienoic C_{18} acids in the total butterfat acids from English pasture butter. The same authors later reported on the spectroscopic analysis of a C_{18} fraction (from butterfat) of which 2·0 % was conjugated dienoic acid (9). Shorland, who found only 1·31 % and 0·51 % of conjugated dienoic acids in the C_{18} fractions of two New Zealand butterfats (19), suggested that there may be a gradual separation of the conjugated from the non-conjugated acids during distillation (20). In contrast to all the findings reported above Schaffer & Holm (21) stated that there was no evidence for the presence of conjugated dienoic acids in American butterfat.

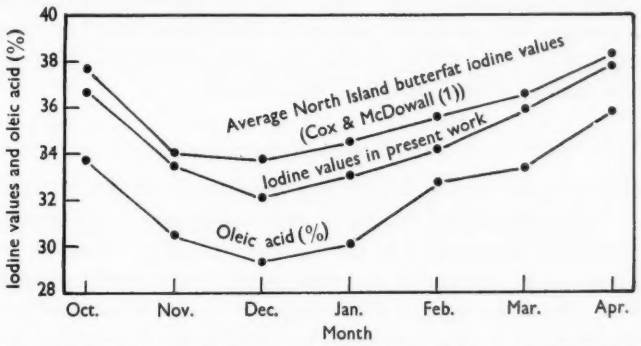

Fig. 1. Average monthly iodine values and average monthly percentage contents of oleic acid in the butterfat examined

Non-conjugated dienoic acids

Average monthly results for the content of non-conjugated dienoic acids fluctuated considerably from month to month, varying from 0·2 to 0·7 %, i.e. somewhat lower than the results quoted by other workers. Hilditch & Jasperson (9) and Shorland (19), for example, found non-conjugated dienoic acids in the C_{18} fractions of the butterfats examined, and on the assumption that the fractions represented one-third by weight of the original butterfats, the non-conjugated dienoic acids in the butterfats would be: 0·7 % (Hilditch & Jasperson); 0·4 % (Shorland, June butterfat); and 1·5 % (Shorland, March butterfat). Hilditch & Jasperson and Shorland concluded that the unconjugated acid was not linoleic but probably an isomer. Mattsson (14) reported the 'linoleic' acid content of Swedish butterfat as varying from 0·8 to 2·0 % with no marked seasonal variations, while Schaffer & Holm (21) reported consistently high figures varying from 2·1 to 2·7 % for the 'linoleic' acid content of American butterfats.

Trienoic acids

The presence of only traces of conjugated trienoic acids in the butterfats examined (Table 1) is in line with the findings for butterfat by other workers (9, 19, 21). Results for

the content of non-conjugated trienoic acids ranged from 0·5 to 1·0%. These figures are of the same order as those (converted to a butterfat basis) quoted by Shorland (19), viz. 1·0% (June butterfat) and 0·7% (March butterfat). Shorland showed also that the octadecatrienoic acid was linolenic acid, and he suggested that the low content (0·2%) of the acid in Hilditch & Jasperson's C_{18} fraction (9) might be due to stall-feeding, whereas the linolenic acid content of New Zealand butterfat from cows on pasture all the year round might always be fairly high. The linolenic acid content of American butterfats (Schaffer & Holm (21)) varied from 0·77 to 1·29% and that of Swedish butter fats (Mattsson (14)) from 0·6 to 2·0%, the higher results occurring during pasture feeding.

It is interesting to note that Mattsson (14), by feeding linseed, which is rich in linolenic acid, found a considerable increase, viz. from 0·5 to 6·7%, in the conjugated dienoic acid contents of the butterfats, thus confirming the earlier work of Dann *et al.* (6) and of Houston *et al.* (7). Mattsson found also that the 'linoleic' acid content fell during the linseed-feeding experiment from 2% to nil, but that linolenic acid remained fairly constant at approximately 1·6%. As the amount of linolenic acid in grass fat is very high (9, 19) it is, perhaps, not surprising to find that butterfats from cows depending almost entirely on a pasture diet, as in the present investigation, have, in comparison with butterfat from stall-fed cows, a high content of both linolenic and of conjugated dienoic acids and a low content of non-conjugated dienoic acid.

Tetraenoic acids

No trace of conjugated tetraenoic acid was found in any of the butterfats examined, but 0·3–0·4% of non-conjugated tetraenoic acid was present in all samples. Bosworth & Sisson (22) recorded the presence of arachidonic acid in an American butterfat, and Mattsson (14) detected the same acid in minute quantities in Swedish samples; but until recently there was no other evidence that tetraenoic acids ever occurred in butterfat. In 1951, however, Shorland & Johannesson (23) reported on the preparation of concentrates of C_{20} and C_{22} unsaturated acids from New Zealand butterfats, and by spectroscopic measurement after alkali isomerization showed the presence not only of tetraenoic but of pentaenoic and even hexaenoic acids. They found that the pentaenoic and hexaenoic acids after alkali isomerization developed maximum absorption at 348 and 375 mμ respectively, and also that both acids developed considerable absorption at 316 mμ—the maximum for tetraenoic acids. Thus, if higher unsaturated acids were present in the butterfats examined, the results reported in Tables 1 and 2 for contents of non-conjugated tetraenoic acids may be too high, due to the non-inclusion of correction for absorptive values for the tetraenoic acids produced from the isomerization of pentaenoic and hexaenoic acids.

SUMMARY

Variations in the unsaturated acid constituents of butterfat samples from the Manawatu district of the North Island of New Zealand over the months October to April were studied by the spectrophotometric technique, applied both before and after alkali isomerization of the fat.

The seasonal variations in the iodine values of the butterfats were found to be closely related to the changes in oleic acid content. Conjugated dienoic acids, though present in relatively small amounts, showed a somewhat similar seasonal trend in values to oleic

acid. The results for percentage content of non-conjugated dienoic acids were low, and they fluctuated considerably from month to month. Conjugated trienoic and tetraenoic acids were absent or present only in traces. Small but fairly consistent quantities of non-conjugated trienoic and tetraenoic acids were found in all samples.

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REFERENCES

(1) Cox, G. A. & McDowall, F. H. (1948). *J. Dairy Res.* **15**, 377.
(2) Hansen, R. P. & Shorland, F. B. (1952). To be published in *Biochem. J.*
(3) Gillam, A. E., Heilbron, I. M., Hilditch, T. P. & Morton, R. A. (1931). *Biochem. J.* **25**, 30.
(4) Dann, W. J. & Moore, T. (1933). *Biochem. J.* **27**, 1166.
(5) Booth, R. G., Kon, S. K., Dann, W. J. & Moore, T. (1935). *Biochem. J.* **29**, 133.
(6) Dann, W. J., Moore, T., Booth, R. G., Golding, J. & Kon, S. K. (1935). *Biochem. J.* **29**, 138.
(7) Houston, J., Cotton, A. G., Kon, S. K. & Moore, T. (1939). *Biochem. J.* **33**, 1626.
(8) Moore, T. (1939). *Biochem. J.* **33**, 1635.
(9) Hilditch, T. P. & Jasperson, H. (1945). *J. Soc. chem. Ind., Lond.*, **64**, 109.
(10) Mitchell, J. H., Kraybill, H. R. & Zscheile, F. P. (1943). *Industr. Engng Chem.* (Anal. ed.), **15**, 1.
(11) Brice, B. A. & Swain, M. L. (1945). *J. opt. Soc. Amer.* **35**, 532.
(12) Brice, B. A., Swain, M. L., Schaeffer, B. B. & Ault, W. C. (1945). *Oil & Soap*, **22**, 219.
(13) Baldwin, A. R. & Daubert, B. F. (1945). *Oil & Soap*, **22**, 180.
(14) Mattsson, S. (1949). *12th Int. Dairy Congr. Stockholm*, **2**, 308.
(15) Report of the Spectroscopy Committee (1949). *J. Amer. Oil Chem. Soc.* **26**, 399.
(16) Hilditch, T. P., Moore, R. A. & Riley, J. P. (1945). *Analyst*, **70**, 68.
(17) Stand. Specif. Brit. Stand. Instn, no. 769 (1938).
(18) Hilditch, T. P. & Jasperson, H. (1941). *J. Soc. chem. Ind., London*, **60**, 305.
(19) Shorland, F. B. (1950). *Nature, Lond.*, **166**, 745.
(20) Shorland, F. B. (1951). Private communication.
(21) Schaffer, P. S. & Holm, G. E. (1950). *J. Dairy Sci.* **33**, 865.
(22) Bosworth, A. W. & Sisson, E. W. (1934). *J. biol. Chem.* **107**, 489.
(23) Shorland, F. B. & Johannesson, D. J. (1951). *Nature, Lond.*, **168**, 75.

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504. STUDIES ON A WASHED CURD VARIETY OF CHEESE

I. THE INFLUENCE OF DIFFERENT TYPES OF STARTER

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(With 3 Figures)

Large-scale manufacture of washed curd cheese has not yet been attempted in Britain. There seems to be no obvious obstacle, and the production of a good washed curd cheese would satisfy several present-day requirements. It is popular with consumers and would be an alternative to the usual standard varieties; it has certain economic advantages in manufacture and excellent properties for retail sale. In addition, low acid whey is in considerable demand as an important ingredient of certain proprietary brands of infant foods; such whey is not readily obtainable by normal cheese-making, but is produced during the manufacture of this cheese.

For these reasons, experimental manufacture and ripening of washed curd cheese has been undertaken as part of a more comprehensive inquiry into the comparative ripening of the Cheddar, Swiss and washed curd varieties.

The washed curd group of cheese, comprising Edam, Gouda, Danish-Swiss, Steppe, Iowa Swiss and other varieties, is characterized by a close plastic body, for which the combined washing and scalding process is responsible. In some respects the cheese may be considered to combine the properties of Cheddar and Swiss types.

METHOD OF MANUFACTURE

Twelve gallons of heat-treated milk were cooled to 90° F., starter was added (in accordance with the experimental procedure described later), followed by 10 g. of potassium nitrate and 6 drams of rennet. The curd was left for 1 hr., cut once each way with horizontal and vertical 'American' knives, and then slightly cross-cut with the vertical knife. Three gallons of whey were removed immediately and replaced with water at 140–150° F.; this process was repeated once more and the temperature adjusted to give a final scald of 103° F. Washing was effected as rapidly as possible and the curd subsequently stirred for 1 hr. After pitching momentarily, it was pushed to one end of the vat, racked and weighted, and the whey run off. The curd was cut to the shape of the mould, moulded and pressed for 24 hr., during which period it was turned three times into clean cloths. It was then placed in 20% brine at 60° F. for 48 hr., and finally taken to a Cheddar ripening cellar, at a temperature of 55–60° F. After ripening for 7 days the cheese was waxed.

Its similarity to Cheddar is evident in the method of cutting the curd and in the final temperature of scald which, in these experiments, was eventually standardized at 103° F. Cheddar-ripening room conditions, at a temperature varying between 55 and 65° F., were found to produce satisfactory cheese, and bacteriological examinations revealed a high proportion of lactic streptococci with a marked similarity to the flora of the English hard-pressed varieties. The bacteriological data will be the subject of a future publication,

but a brief summary of the types found in four cheese (Table 1) gives a general indication of the flora.

The closeness and plasticity of the ripened cheese body bears a marked resemblance to that of the Swiss varieties; this closeness of body is probably responsible for separation of the gas produced during ripening in the form of bubbles. In a few experimental cheese the gas holes were large, round and shining, exhibiting an additional similarity to those produced regularly in Swiss cheese. These similarities of body between the two types are of interest, since they are produced by entirely different methods of manufacture.

A lactic starter is always used in the manufacture of Dutch, Danish-Swiss, Steppe and Iowa Swiss cheese, but a Swiss fermentation has been encouraged by the addition of propionic bacteria with the starter in the Iowa Swiss variety (2). The usual combination of starters used to ensure good ripening in a Swiss cheese is a mixed culture of *Lactobacillus helveticus*, *Streptococcus thermophilus*, *L. bulgaricus* and *Propionibacterium shermanii* (3). It was thought that a mixed starter of *Str. thermophilus* and *L. helveticus* in place of a lactic culture might give the low acid conditions suitable for the production of those Swiss properties which are desirable in a washed curd cheese. The point was investigated on Trappist cheese (4), and a lactic starter was found to be more satisfactory.

Table 1. *Numbers of lactic streptococci and other types in samples of four cheese*

Age ...	1–3 days				4–5 days			12–19 days				31–45 days				49–63 days	
Cheese no....	85	86	87	88	85	87	88	85	86	87	88	85	86	87	88	85	86
Str. lactis	804	815	695	669	774	659	—	334	184	421	—	92	326	—	—	148	111
group	802	821	694	664	778	—	—	—	208	—	—	190	—	—	—	—	—
All other	—	—	—	—	—	—	774	334	808	218	370	559	398	504	503	495	569
types	—	—	—	—	—	—	—	—	762	405	—	470	—	453	481	—	—

The popular American variety, Brick, may, or may not, be made by using a washing method (5). Where washing is employed, it is reported that a more desirable mild flavour, together with a smooth, soft, medium close body, is obtained. In this case, washing takes place immediately before dipping, so that the curd is subjected to the water treatment for only a short time. A lactic starter is used, and the cheese may conveniently be considered as intermediate between the Cheddar and the washed curd varieties.

This investigation, together with other experiments on standardization of the manufacture of this cheese, was planned to establish the most desirable method for use in Britain. The experiment to be described was designed to study any advantages or drawbacks associated with the use of a Swiss instead of a lactic starter.

PREPARATION OF STARTERS

(*a*) *Lactic*

Several different mother strains of *Str. lactis* were propagated daily in fresh whole milk which had been held at 202° F. for 1 hr. and cooled to 85° F. The titratable acidity developed in 24 hr. varied from 0·9 to 1·3% lactic acid. The starter was characterized by quick acid production with no sign of gas or off-flavours. The quality of the starter was assessed by the acidity produced after growth at 70° F. for 24 hr., and by the speed of working in vats of Cheddar cheese which were being made at the same time and with the same starter as the washed curd cheese. A 2% inoculation of a mixture of the mother cultures was used.

(*b*) *Swiss*

Commercial powder cultures, described as '*Thermococcus*' and '*Bact. casei* E' were used: they corresponded to Bergey's *Str. thermophilus* and *L. helveticus* respectively. *L. helveticus* is considered essential for making Emmenthal cheese, while additional amounts of *Str. thermophilus* starter, varying with the acidity and general quality of the milk, are also recommended.

Both starters were renewed at frequent intervals, since the *Str. thermophilus* culture is particularly subject to overgrowth by contaminants. On one occasion it became contaminated with a rapid acid-producing organism, and in another instance a very pronounced taint which was not associated with any change in acidity made it necessary to discard the starter. The *L. helveticus* culture always showed considerable gas formation during the initial inoculations. After a few subculturings this property disappeared. Both starters were incubated at 72° F., and although higher inoculation temperatures are advised, yet acid production and coagulation did not appear to be adversely affected (6). The *Str. thermophilus* culture was removed from the incubator after 6 hr. growth. The *L. helveticus* culture was maintained at 2·6–3·6% lactic acid, and the *Str. thermophilus* culture at 0·45–0·75% lactic acid after 24 hr. growth.

Inoculation into cheese milk

(*a*) *Lactic.* A 1% inoculation was used. Fisker (1) states that this gives a suitable curd for the manufacture of the Danish-Swiss and Steppe varieties.

(*b*) *Swiss.* A 1% inoculation of the *L. helveticus* milk culture was used, together with 6 ml. of *Str. thermophilus* culture per 12 gal. of cheese milk.

In both cases the starter was added to milk at 85° F., and the milk was rennetted immediately without a ripening period.

Manufacturing methods

(*a*) *Milk.* The quality of the milk varied from day to day, both chemically and bacteriologically. All the milk was bulked for heat treatment and then divided equally between two vats. One vat received lactic starter and the other Swiss starter, otherwise the treatment was identical. Since the aim of the experiment was to predict the probable reaction of starters under factory conditions, the variability of milk quality was not a disadvantage. The milk was heat treated at 150° F. for 10–14 min. and cooled immediately to rennetting temperature (90° F.).

(*b*) *Cheese-making.* The method outlined in the introduction to this paper was adhered to throughout the course of the experiments.

RESULTS

Certain terms have been used to describe qualities of body, flavour and eyehole formation in the cheese when cut for grading. Tabulation of these results proved difficult. For example, a cheese flavour described originally as 'Swiss, slightly bitter'—thus indicating that it is acceptable for marketing requirements, but defective by highly critical judgement—must be entered as both 'Swiss' and 'bitter'. This method of analysis seems best suited to the nature of the data, but has the disadvantage of precluding statistical treatment.

All the examinations and conclusions were made by one person. This was inevitable since the cheese were cut over a period of at least 2 years, and since the final comparisons are based on these judgements only general conclusions can be drawn. The full descriptions of each cheese are too bulky for inclusion here and only final grouped analyses are given.

(*a*) *Body.* Variations from a good body were not frequent and were easily recognizable. The data are given in Table 2. If the descriptions 'very good', 'good' and 'fairly good' are combined as 'satisfactory', this category would include 75% of the lactic and 70% of the Swiss starter cheese.

(*b*) *Flavour.* The original detailed descriptions of flavour have been reduced to the following five headings: mild, lactic, 'Swiss', bitter, meaty.

The description 'Swiss' refers to the characteristic sweet flavour which is associated with Gruyère and Emmenthal and is entirely absent from the English varieties. The propionic acid bacteria are responsible for this flavour (7, 8).

Table 2. *Grading of experimental cheese*

Body					Flavour					Eyehole formation				
	Lactic starter		Swiss starter			Lactic starter		Swiss starter			Lactic starter		Swiss starter	
Grade	No.	%	No.	%	Grade	No.	%	No.	%	Grade	No.	%	No.	%
Very good	7	35	7	31	Lactic	13	32·5	8	21	Good	7	28	8	28·6
Good	7	35	8	34	Mild	15	37·5	10	26·5	Round	2	8	8	28·6
Fairly good	1	5	1	4	Swiss	6	15	8	21	Irregular	5	20	1	3·5
Rough	2	10	2	10	Bitter	3	7·5	12	31·5	Flat	1	4	—	—
Tough	3	15	4	17	Meaty	3	7·5	—	—	Ragged	6	24	7	25
Soft	—	—	1	4						Blown	4	16	4	14·3
Total	20		23			40		38			25		28	

'Lactic' in this analysis is descriptive of a clean, fresh flavour, not strongly acid but generally associated with a lactic rather than a Swiss fermentation, and typical of the flavour of a good Dutch cheese.

'Meaty' flavour is indicative of a putrid type of proteolysis, undesirable in any cheese.

'Mild' indicates that the cheese was ripening satisfactorily, but the flavour had not developed sufficiently.

The results are presented in Table 2. If 'Swiss', mild and lactic are considered as satisfactory for market requirements, the classification becomes:

	Lactic (%)	Swiss (%)
Satisfactory	85	68·5
Unsatisfactory	15	31·5

Bitterness in Cheddar cheese has been associated with a low salt content (9). Burkey, Rogosa & Farrar (10) found the same relationship in Swiss cheese; the organisms responsible for the bitter flavour were found to be *Bacillus vulgaris* and *B. mesentericus*, and their growth was inhibited by heavy salting. *Micrococcus* species (11) have also been reported as responsible for bitterness in Cheddar. Degrees of bitterness were recorded from 0 to 7, and grades 0, 1 and 2 would be considered satisfactory for commercial purposes. The much higher incidence of bitterness in the Swiss starter cheese (Table 2) suggests that use of this particular starter may be significant. It was thought that either lessened activity of the cultures or a high acidity of the *L. helveticus* starter might possibly be responsible for this defect. Fig. 1 shows the relationship between the acidity of the starter and the

development of a bitter flavour, and indicates that the defect is probably not related to starter activity. The increasing incidence of bitterness with the age of the cheese is, however, shown in Fig. 2.

A 'meaty' flavour was the only other outstanding defect. It was generally associated with excessive hole formation and sometimes with a grey colour. In early experiments the organism responsible appears to have been a fairly common contaminant. Bacteriological examination of a cheese exhibiting this defect showed large numbers of *B. megatherium* at 3 days old. The organisms were isolated by plating sodium citrate dilutions of the emulsified cheese on dextrose-yeast malt agar, and were identified by their cultural reactions according to Bergey's classification (12).

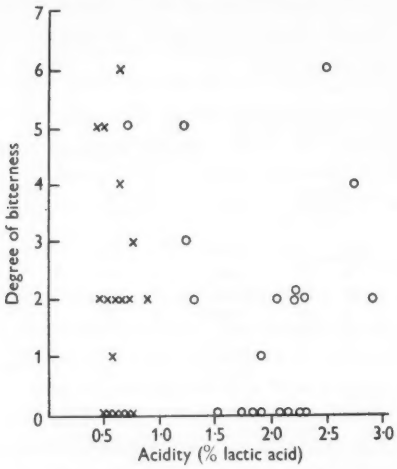

Fig. 1. × *Str. thermophilus;* ○ *L. helveticus.*

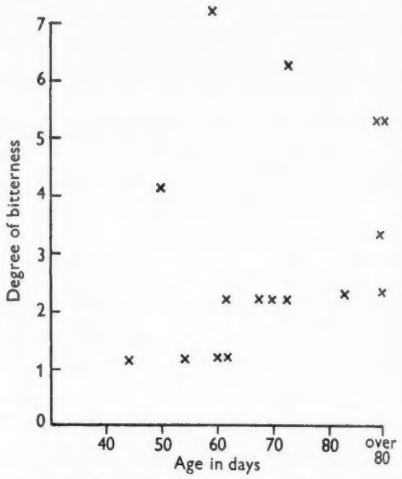

Fig. 2. Effect of age on degree of bitterness.

(c) *Eyehole formation.* Tracings of the cut cross-section of the cheese were prepared throughout the experiments and enabled close comparisons to be made.

The data (Table 2) for eyehole shape were grouped under six headings: good (which included very good and fairly good), round, irregular, flat, ragged and blown. If the groups 'good' and 'round' are considered as satisfactory, the following results are obtained:

	Lactic (%)	Swiss (%)
Satisfactory	36	57
Unsatisfactory	64	43

GENERAL CONCLUSIONS AND DISCUSSION

The results of this survey of the manufacture and ripening of a washed curd variety of cheese relate mainly to the practical aspects of the problem. This paper deals only with the single aspect of the use of starter and the data yield suggestions rather than definite conclusions. Nevertheless, one or two interesting points have emerged. No advantage in respect of cheese body was to be gained by using a Swiss starter. Variations were within small limits and the judgements were highly critical. Faults of body occur only rarely, and these are generally associated with abnormal variations in scalding temperatures and washing procedure.

A significantly higher number of good-flavoured cheese resulted when a lactic starter was used, so that in general it is considered more satisfactory for the manufacture of this type of cheese. Bitterness and meatiness were more evident in cheese made with Swiss starter. Bitterness was found to be unrelated to the acidity of the starter, but showed a general tendency to increase with ageing of the cheese. The typical Swiss flavour characteristic of Gruyère and Emmenthal occurred more often when Swiss starters were employed, although it is of interest to note that the flavour was sometimes produced in the absence of a Swiss starter.

The Swiss starters produced, on the whole, a considerably better type of eyehole than the lactic starter, when the cheese were judged according to the standards usually applied to the Swiss varieties. The question arises, however, whether these standards should be applied in judging a washed curd variety of cheese. Comparisons of the characteristics of different types of washed curd cheese are difficult, since they can only be made

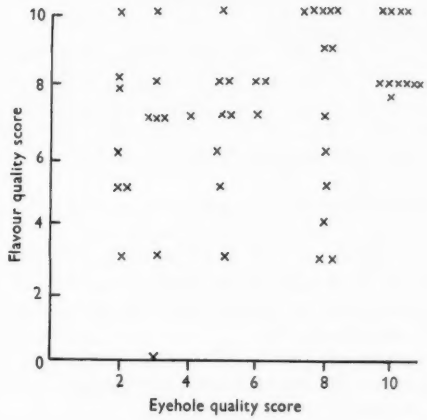

Fig. 3

through the literature. As far as can be judged they can be grouped according to their flavour characteristics into (*a*) those possessing a sweet Swiss flavour, (*b*) those exhibiting the clean lactic flavour typical of the Dutch varieties.

The Swiss flavour is in general associated with the presence of the propionic acid bacteria (9), and from the literature it would appear that they are not essential, though they may be present in Edam, Gouda and other washed curd varieties (13, 14, 15). When, in these experiments, a mixed starter of *L. helveticus* and *Str. thermophilus* was used, the resulting cheese showed a definite increase of 'Swiss' flavour; this indicates that the propionic acid bacteria are encouraged by this particular combination of starters. In general, however, the flavour was not improved. Similarly, Tonka (4), working with Trappist cheese, found that *Str. thermophilus* and *L. helveticus* starters sometimes lowered the grade for taste and smell. He preferred to use a 0·2 % inoculation of butter culture. Thomé (16) also found that a mixed starter of streptococci and betacocci in the proportion of 10:1 produced Swedish Manor cheese with good eyes and fairly good flavour. A more constantly good standard for flavour (but similar to that of Dutch cheese) has been obtained in these experiments by the use of lactic starters.

Since better results for eyehole formation were obtained when Swiss starter was used, the degree of correlation between eyeholes and flavour should be considered. A scatter diagram (Fig 3) indicates that where a score of less than 5 is associated with poor eyes, and over 5 with good flavour, in as many as 25% of the samples good flavour was associated with poor eyes. This suggests that some forms of holing (e.g. irregular, ragged and small holes) which would be deleterious in Swiss cheese, are not necessarily associated with poor flavour qualities in washed curd cheese. The eyehole results cannot, therefore, be considered indicative of good quality; the flavour judgement appears to be the most important.

On these grounds, the use of a lactic starter seems preferable. In addition, the Dutch type flavour is probably more popular in Britain, and from a commercial point of view the propagation and maintenance of the lactic starter would be easier to accommodate in a British factory.

REFERENCES

(1) Fisker, A. N. Personal communication.
(2) Babel, F. J. & Hammer, B. W. (1939). *Res. Bull. Ia Agric. Exp. Sta.* no. 264.
(3) Frazier, W. C., Sanders, G. P., Boyer, A. J. & Long, H. F. (1934). *J. Bact.* **27**, 539.
(4) Tonka, G. (1947). *Tejgazdasag*, **5**, 145. Hung. Exp. Inst. Mosonmagyarovar.
(5) Foster, E. W., Garey, J. C. & Frazier, W. C. (1942). *J. Dairy Sci.* **25**, 313.
(6) *Ann. Rep. Agric. Exp. Sta. Univ. Wisconsin* (1943), no. 461, 38.
(7) Sherman, J. M. (1921). *J. Bact.* **6**, 379.
(8) Babel, F. J. (1939). *Iowa St. Coll. J. Sci.* **14**, 6.
(9) Tuckey, S. L. & Ruehe, H. A. (1940). *J. Dairy Sci.* **23**, 517.
(10) Burkey, L. A., Rogosa, M. & Farrar, R. R. (1940). *J. Dairy Sci.* **23**, 513.
(11) Evans, A. C., Hastings, E. G. & Hart, E. B. (1914). *J. agric. Res.* **2**, 167.
(12) *Bergey's Manual of Determinative Bacteriology* (1948), 6th ed., p. 714. London: Tindall and Cox.
(13) Kheraskov, S. (1940). *Mol. Prom.* **7**, 10.
(14) Demeter, K. J. & Janoschek, A. (1941). *Zbl. Bakt.* II, **103**, 257.
(15) Van Beynum, J. & Pette, J. W. (1943). *Milchw. Zbl.* **70**, 252.
(16) Thome, K. E. (1946). *Svenska Mejeritidn.* **38**, 513, 523.

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637.128:616.9

REVIEWS OF THE PROGRESS OF DAIRY SCIENCE

SECTION F. MILK-BORNE DISEASES

GENERAL

The statistical data on which evidence of milk-borne infection is built up is frequently unsatisfactory. The returns of such infections are often very incomplete, and it is highly probable that many cases occur without being brought to the knowledge of the authorities. A survey of the literature of the period since the last review was published in 1949 suggests, however, a lessening of the incidence of some milk-borne diseases for, presumably, the continued increasing supervision, through all its stages, of the milk-producing industry, makes milk a less likely vehicle for carrying infection. Furthermore, Feemster(1) has been reporting on the prevalence of milk-borne disease in Massachusetts since 1919, and the present paper brings the story up to the end of 1949. He stated that no important outbreaks have been notified during the 5-year period under review, though two outbreaks of gastro-enteritis, probably due to a staphylococcal enterotoxin, occurred, but these outbreaks were not proved to have been due to infected milk. He noted that in 1919 only 34% of the milk was pasteurized, but in 1949, 99% of the milk to 168 communities was so treated, and 93% of the whole population of this state received such milk. Savage(2) reviewed milk-borne outbreaks in Great Britain and summarized recent literature on this subject. He described the various sources of infection and called attention to the fact that these might be primarily bovine, or secondary to infection of the cow's udder with human pathogens, or due to direct contamination of milk by humans at some stage in the handling of it.

In this country a great deal has yet to be done to make milk safe, but a rapid increase has taken place in the numbers of catttle in tuberculin-tested herds, and with the introduction of 'specified' areas and extensions of eradication schemes better results may still be anticipated. Kay(3) discussed the report of the Joint Committee of the British Medical and the National Veterinary Medical Association on the provision of an adequate and safe milk supply. He stated that few would disagree with the necessity for safe milk, and he hoped that the Minister of Health would lose no time in promoting orders by which only designated T.T. or pasteurized milk will be sold in specified areas. Tullis(4) and Gibson(5) discussed the Milk and Dairies Regulations 1949, and the Milk (Special Designations) (Raw Milk) Regulations, 1949, which apply to England and Wales. These new measures were designed to increase further the supply of safe milk to the public. The first regulation gives the Medical Officer of Health powers to stop the sale or to order milk to be heat-treated if he suspects it to be infected with any disease-producing agents, bovine or human, which can be communicated to man. It is pointed out, however, that these new regulations still fail to give effective control over the producer-retailer of non-designated milk. Local authorities did supervise the production of milk on farms, but this is now a function of the Ministry of Agriculture. Sterilized milk is now recognized as a grade, pasteurized milk must be sold in bottles after 1950 and must be filled only at the pasteurizing plant after 1954. In Scotland the Milk (Special Designations) Act, 1949, was designed to come into force on 1 April 1952. Under this Act the Minister of Food is empowered to specify areas within which milk sold by retail must either be obtained from tuberculin-tested cows, or made safe by pasteurization. The Milk (Special Designations) (Scotland) Order, 1951, which came into force on 1 June 1951, provides for the early discontinuance of the designations 'Heat Treated' and 'Standard', and also provides that after 1954 'Certified' and 'Tuberculin-tested' licences will be granted only for fully attested herds.

In a recent report issued by the Joint WHO/FAO Expert Group on Zoonoses(6), a list of diseases naturally transmitted between vertebrate animals and man is given. This list contains more than eighty diseases, and the report emphasizes the fact that veterinary public health is

an essential service in any community, and that an efficient service promotes the prevention of disease and in consequence increases the efficiency of man. In so far as animals producing milk on a large scale for human consumption are concerned the list includes the following:

Animal	Disease	Conveyed by milk direct from animal (authors comment)
	(1) Virus diseases	
Ruminants	Aujeszky's disease	No
Cattle	Cowpox	No
Ruminants	Foot and mouth disease	Occasionally
Cattle	Vesicular stomatitis	No
	(2) Rickettsial diseases	
Cattle	Q fever	Yes
	(3) Bacterial diseases	
Cattle	Anthrax	No
Cattle and goats	Brucellosis	Yes
Cows	Diphtheria	Yes
Cows	Leptospirosis	Yes
Cattle	Listeriasis	No
Ruminants	Pasteurellosis	No
Cattle	Salmonellosis	Yes
Cattle	Streptococcosis	Yes
Cattle	Tuberculosis	Yes
Cattle	Vibrio abortion	No

The above list, moreover, does not take into account the disease spread through the agency of milk after being infected directly by man.

TUBERCULOSIS

The Report of the Ministry of Health (7) for the year ended 31 March 1950 pointed out that during 1949 the deaths from all forms of tuberculosis showed a definite decline as compared with the totals for 1948; also that mortality from non-respiratory forms of tuberculosis has declined markedly in the last 11 years. It was calculated, however, by the method described by Wilson (8) that for the years 1948 and 1949, 900 adults and 650 children had died from infections due to the bovine type of bacillus.

Alarmed at the apparent increase in the death-rate from tuberculosis during the first 2 years of the Second World War, the Ministry of Health asked the Medical Research Council to investigate the extent and causes of the increase, and to advise on possible preventive measures. This report was issued in 1942 and afterwards certain members of the Committee undertook to extend the investigation in order to find out how much of the increased death-rate from non-pulmonary tuberculosis was ascribable to infection with the bovine type of bacillus. This further report (9) was issued in 1949 and gave particulars for England and Wales separately. The samples for examination were collected in England during 1943, 1944 and 1945 and for Wales during 1944, and the main findings are recorded in Table 1 compiled from these reports. Comparing the English and Welsh figures the proportion of bovine-type infections in non-pulmonary tuberculosis was lower in Wales than in England. The authors pointed out that two or three factors could be responsible for this difference, but undoubtedly one important factor was the much higher proportion of cattle in attested herds in Wales than in England, 21·3 to 5·4 %. Considered in conjunction with the fairly high proportion of milk pasteurized or heat-treated in the larger towns of south Wales, this could probably be taken to indicate that the milk supply to the public was less often infected in Wales than in England. By applying the percentages obtained for the various types of infection the authors estimated that 1300 individuals in England and fifty in Wales died from non-pulmonary tuberculosis of bovine origin. Comparing these figures with estimates made by previous workers, and with the percentages of bovine type infections given by Griffith in 1938, an apparent, though slight, reduction in the amount of disease caused by the bovine type of bacillus had occurred. Chalmers (10) reviewed the position in Scotland, and stated

that the action which was being taken to reduce the incidence of bovine tuberculosis in Scotland was progressing satisfactorily. At 30 September 1949, 38·9% of the cattle in Scotland were attested, which compared favourably with England in which there were 12·8, and in Wales where the percentage was 29·4. Some areas of the country have progressed more rapidly than others. Thus all cattle in Zetland were attested, in Bute 92·2%, Ayrshire 90·2% and Dumfries 75·9%. These herds, however, were not all dairy herds, but progress in the elimination of bovine tuberculosis in such herds was equally satisfactory. In 1939, 32·8 million gallons of 'certified' and T.T. milk were produced, whereas in the first 9 months of 1949, 130·1 million gallons were available.

Francis(11) discussed the control of infection with the bovine tubercle bacillus both in animals and man. He pointed out that though only 18·20% of all cattle in Great Britain react to tuberculin, all these have to be regarded as infectious. Scandinavian work has indicated that the most serious form of bovine pulmonary tuberculosis in man is due to inhaling the infection from cattle. In country districts in Great Britain there is an increased ratio between the incidence

Table 1. *Non-pulmonary tuberculosis*

	England	Wales
Patients examined	994	112
No. infected with bovine T.B.	216	19
Percentage infected with bovine T.B.	26·3	17·0
Percentage of bovine infections:		
(1) Meningitis	28·0	10·1
(2) Cervical adenitis	57·5	44·4
(3) Bone and joint	10·6	19·0
(4) Abdominal	64·7	—
(5) Genito-urinary	22·5	—
(6) Other sites	11·8	—
Percentage of individuals infected with bovine type:		
Under 15 years	40	22·4
15 years and over	15	11·1
History of exposure to open pulmonary infections:		
(1) Human, percentage	25·8	18·4
(2) Bovine, percentage	9·4	6·7
History of habitual milk drinking:		
(1) Bovine infection, percentage	86	80
(2) Human infection, percentage	53·0	55·4

of bovine tuberculosis and the total mortality from human tuberculosis. Evidence from Denmark indicates that where tubercle bacilli are present in the milk the number of human reactors to tuberculin is greater in these areas than in areas where there is complete pasteurization. On the other hand, there is more actual tuberculous disease in the human in those areas where there are no tubercle bacilli in the milk. He suggested, therefore, that as milk is freed from tubercle bacilli by the eradication of tuberculosis in cattle and by pasteurization, more will have to be done in the way of immunizing humans against tuberculosis with B.C.G. Furthermore, as bovine tuberculosis is eradicated the risk of cattle becoming infected from man will increase. In another paper Francis(12) has examined the various aspects of the tuberculosis problem, bacteriology, comparative pathology, eradication schemes, pasteurization and immunization. Attention is called to the relationship of nutrition to tuberculosis, particularly in India.

Reilly(13) reported from Northern Ireland that after an examination of specimens of sputum from 1060 cases of pulmonary tuberculosis only one bovine infection was found. In specimens from 364 cases of non-pulmonary infection the percentage of the bovine type was 4·3, while that for tuberculous meningitis alone was 3·3. In the same region, Kerr, Lamont & McGirr(14) carried out a post-mortem examination of 600 cows and found that 201 had some form of tuberculosis, representing an incidence of 33%. Also, approximately 7·5% of churn samples of milk contained tubercle bacilli, and that, therefore, the chances for infection were still fairly

numerous. They concluded that the pattern of tuberculous infection is changing and the mortality rate in all civilized countries is decreasing, and that a major factor in eliminating bovine-type infection is pasteurization. Hedvall(15) reported that bovine tuberculosis is now rapidly diminishing in Sweden, and in all probability will disappear entirely in the near future. Laws forbidding the sale of milk other than pasteurized have been enacted, and cattle are under continuous inspection and control by the state. Meara(16) examined 1174 samples from herds in the Johannesburg area by guinea-pig inoculation and found that only 1·02% contained tubercle bacilli, while 16·1% of 217 samples showed the presence of *Brucella abortus*. Henning & van Aswegen(17) obtained composite samples of milk from 200 herds also in South Africa and had them examined by guinea-pig inoculation. Eight samples or 4% were found to contain tubercle bacilli.

Attempts have been made to use recently introduced types of media for the cultivation of tubercle bacilli in milk. Thus Paterson & Winmill(18) tried, in a series of forty-six milk samples diagnosed by microscopic examination of stained smears as being from cows with udder tuberculosis, to culture the organism in Dubos medium. Each milk sample was tested on Dorset's egg, Lowenstein-Jensen and Dubos media. The experiments demonstrated that the use of Dubos's liquid medium, combined with oxalic acid for destroying contaminants, failed to detect minimal numbers of tubercle bacilli still capable of infecting guinea-pigs, but gave much better results than solid media. Kästli & Binz(19) carried out tests on the viability of tubercle bacilli in different types of cheese, infected milk being used for this purpose. In hard types of cheese, such as the Emmental and Gruyère varieties, the tubercle bacilli survived from 5 to 30 days, in semi-soft cheese for 305 days, and in soft Camembert up to 47 days. The organisms were demonstrated in the samples by means of guinea-pig inoculation.

SALMONELLA INFECTIONS

(1) *Serological classification*

Kauffmann(20), in a recent book on the entero-bacteriaciae, has shown that this group of organisms with sixty-two 'O' antigens, H antigens *A* to *Z*, Z_1 to Z_{36}, and 1–11 now comprise 209 types, and since this volume was published a few more have been added. The necessity for *Salmonella* reference laboratories in London, Copenhagen and the U.S.A. is, therefore, apparent if order and accuracy in type determination is to be maintained. As regards the typhoid and paratyphoid members of the group, Felix & Anderson(21) now recognize twenty-nine Vi-phage types of *S. typhosus* and four types of *S. paratyphosus* B.

(2) *Enteric fevers*

A W.H.O. Report(22) summarizes the statistics of the incidence of typhoid and paratyphoid fevers since 1947. In Europe there has been a general decrease in cases in most countries with the exception of France and Spain. In England and Wales there was a record low incidence in 1949, when there were 280 cases of typhoid and 560 cases of paratyphoid. In most parts of Africa there was also a decline, but in Northern and Southern Rhodesia, Egypt, Kenya and in the Union of South Africa there has been a considerable increase. In Central America there has been a general decline, while in the United States the incidence is again extremely low.

Sartorius(23) gave an account of a typhoid epidemic which occurred in Potsdam. There, 132 cases of verified typhoid fever, forty-eight suspected, and thirty-eight unconfirmed, were encountered and in the neighbourhood there were seventy cases of verified, two suspected, and twenty-one unconfirmed, eighteen of which proved eventually to be paratyphoid. Women and children were mostly affected, and although not definitely proved, milk was suspected as the source. Evidently the arrangements for pasteurization of the milk had been unsatisfactory, and so also administrative control and hospitalization. Werner & Zöckler(24) reported the occurrence at Kamberg in Germany of a large outbreak of paratyphoid B fever due to milk. Prior to the main outbreak a series of sporadic cases had been notified, but by the time the epidemic had

ended about 1000 cases were estimated to have existed. The central dairy supplied twenty-five villages and the inhabitants in twenty-one were affected. Further, a considerable amount of milk was sent daily to Weisbaden and many further cases occurred there. Examination of the staff of the dairy, twenty-five in all, failed to show any carriers, but it was found that a woman who lived in a flat above the dairy garage had had paratyphoid. It was considered that she was the source, but apart from having contact with the staff of the dairy and using the same lavatory, she otherwise had no association with the distribution of milk. Furthermore, it is not certain whether or not the milk was infected on repeated occasions, or only once, and as the milk was heated to 85° C. for 30 sec. the difficulty of explaining how the milk became infected is apparent. Cockburn, Jamieson & Fenton(25) described an outbreak of paratyphoid B fever at Eastbourne involving fifty individuals, of whom forty-six were primary cases. The vehicle of infection was cream in cream cakes, but how the cream became infected was not fully determined. Although thirty-three members of the staff of the bakery were examined only one was found to have paratyphoid organisms in his faeces, but this man's history suggested that he was one of the primary cases and not the source of infection. The phage type of the organism causing the epidemic was 'Taunton'.

(3) *Food poisoning*

A report in the *Monthly Bulletin of the Ministry of Health and the Public Health Laboratory Services* (26) gave an analysis of food-poisoning outbreaks during the years 1941–8. In all, 3495 incidents were noted, and *Salmonella* organisms were associated with 3193, or 91%. Milk was the vehicle of infection in only fourteen. Smith & Buxton(27) carried out an investigation into the incidence of Salmonellae in the faeces of domestic animals in England. They obtained 750 specimens from normal cattle and of these three, or 0·4%, contained *S. dublin*. Five

Table 2. *Faeces and bile from individual cattle yielding* Salmonella dublin

	Faeces (separate beasts) specimens			Bile (separate beasts) specimens		
	Total	Positive	Percentage positive	Total	Positive	Percentage positive
Irish	1254	126	10·0	116	4	3·4
Non-Irish	855	32	3·7	218	1	0·5

hundred specimens were also received from diseased cattle, and again three, or 0·6%, contained *S. dublin*. No other members of the *Salmonella* group was encountered. In the faeces from 100 diseased goats not a single pathogen belonging to this group was found. Specimens from farmyard poultry showed a much higher incidence and greater diversity of types than cattle. An investigation into the incidence of *S. dublin* in healthy cattle has been carried out by Ritchie & Clayton(28) in Birkenhead, which is one of the largest slaughtering centres in the country. Samples of faeces before and of bile and liver after slaughter have been examined. During the period 1 November 1949 until 31 December 1950, 2109 samples of faeces and 334 of bile were examined; of these only one specimen yielded *S. typhi murium*, but 158 specimens of faeces and five samples of bile were positive for *S. dublin*. The Irish animals investigated were chiefly bullocks and the non-Irish animals bullocks and cows, the latter in particular being discards from dairy herds. The *S. dublin* type was distributed as is shown in Table 2. From August 1950 until the end of the year, 762 milk deposits were also cultured, but only one sample of a pooled milk from the Shropshire-Cheshire-Staffordshire border was found to be infected. Taylor(29) described her experience at the Central Public Health Laboratory of *Salmonella* infections in man and animals during 1947, 1948 and 1949. The figures in the various tables were compiled after typing strains sent for identification and, therefore, they may not give an accurate picture of Salmonellosis in the animal kingdom, but they do indicate to a certain extent the type of animal from which strains can be isolated. The organisms isolated from bovines were *S. typhi murium* (11), *S. thomson* (2), *S. dublin* (167), *S. give* (1), *S. brancaster* (1) and *S. derby* (5). Out of

a total of 3495 incidents due to all types of infected food *S. typhi murium* was associated with 2284 and *S. dublin* with 102. The report of a milk-borne food-poisoning attack due to *S. typhi murium* appeared in the *Lancet*(30). The epidemic affected 332 schoolchildren who drank milk obtained from two farms, S and D. The peccant milk was identified by the fact that part of the supply from farm S went to another district and no illness was caused through drinking this. The cowman at farm D had been ill with diarrhoea and vomiting during the week previous to the outbreak but had said nothing. From his faeces, from milk obtained from the farm, and from the faeces of schoolchildren *S. typhi murium* was isolated. Cromb & Murdock(31) and Moore & Melaney (1949) gave an account of an outbreak of food poisoning due to *S. dublin*. The outbreak occurred at Castlederg, County Tyrone, Eire, and fifty persons were involved. All the cases were associated with raw milk supplied from a particular farm dairy, which obtained its supplies from two sources, the fresh milk from the farm and pasteurized milk from another source. A cow on the farm had been taken ill and had been milked following the onset of the illness. Four days later she died of scour and in the meantime the epidemic had occurred. From specimens from this animal, from faeces of patients, and from the faeces of another animal on the farm, *S. dublin* was isolated.

BACILLARY DYSENTERY

Only one report of an outbreak of bacillary dysentery due to milk has been found in recent literature. Hemmes & van Wesemael(32) described an epidemic of Sonne dysentery which occurred in Holland. The outbreak lasted about a week and some 125 individuals were involved, without, however, any fatal results. The outbreak was apparently due to the contamination of the milk and ice-cream at a single factory by a workman who was found to be a carrier. This man was employed in adjusting the percentage of cream in the milk after it had been pasteurized and had infected it.

DIPHTHERIA

Although outbreaks of diphtheria due to milk are comparatively infrequent in their occurrence a considerable number have been reported. Presumably milk can become contaminated with *Corynebacterium diphtheriae* by two methods. First, by direct contact with infected human beings and secondly by the implantation by human beings of the organisms on surface lesions of the udder and then contamination of the milk from this source. The greater the prevalence of cases and carriers the greater must be the possibility of milk infection. Now, with the incidence of diphtheria at a minimum, no record of the occurrence of an epidemic due to this organism can be found in the literature which has been examined for the purpose of the present review. MacQueen(33) has noted that the number of cases and deaths from diphtheria in Scotland has fallen steadily for the past 7 years, since the start of the immunization campaign. Cases in 1949 were only 7% of the total of 1941, and deaths were under 6% of those for the same year.

STREPTOCOCCAL INFECTIONS

A study of the epidemiology of group A streptococcal milk-borne infections has shown that in most epidemics the sequence of events was an infection of the workers followed by a transference of a human strain to the udder of a cow and then the occurrence of a mastitis in that animal.

It has long been known that cow's milk has the ability to inhibit the growth of human types of haemolytic streptococci. It was shown by Jones and his co-workers(34,35,36) that the milk contained a heat-labile anti-streptococcal substance which they named 'lactenin'. This substance has been reinvestigated recently by Wilson & Rosenblum(37,38,39). These workers have now shown that the measurement *in vitro* of lactenin is affected by the size of the inoculum, the temperature of incubation, and the type of medium employed. The various serological groups of streptococci vary in their sensitivity, but all group A strains regardless of their type are highly sensitive to it, and milk containing haemolytic streptococci receiving a small inoculum sterilizes itself within 48 hr. or less. They have also shown that lactenin is inactivated by the exclusion of atmospheric oxygen and that group A streptococci, when subjected to its action, are not merely

prevented from multiplying but actually killed. The ability of lactenin to prevent the multiplication of group A streptococci when milk becomes contaminated with that organism accounts in part for the infrequency of milk-borne epidemics. Lactenin fails to prevent the establishment of mastitis due to group A streptococci because the milk in the cow's udder is at a low oxidation-reduction potential and this substance is, therefore, inactive.

On the other hand, in the two epidemics which are now described no infection of the cows could be found. Farnback(40) described a milk-borne epidemic of scarlet fever which occurred at the small town of St Arnaud, north-west of Melbourne, in Australia. The population of this town was some 3000 individuals, and between August and September 1949 fifty cases of scarlet fever were notified, and in addition about as many individuals suffered from sore throat without having a rash. Those affected had mostly received their milk supply from a common source. The forty-five cows in this dairy farm and sixty-eight in two other farms were examined and nine showed evidence of mastitis, but group A strains of streptococci were not recovered. At the farm which provided most of the milk to those affected, group A strains were isolated from throat swabs taken from two children and an adult worker, and also from a sample of milk. When the milk was stopped the epidemic ceased. Schoechli(41) described an epidemic which occurred in the Swiss town of Aarau involving 192 persons. The epidemic began suddenly just after Easter, 1949. Two out of six individuals who had been working in the dairy were found to have had an attack of scarlet fever, and when the supply was withdrawn the epidemic began to decline. Bacteriological examination of the milk from the cows failed to show haemolytic streptococci. An outbreak of scarlet fever in Lanarkshire which was presumed to be due to infected ice-cream was reported on by Reekie(42). In all, ninety-seven individuals, thirty-nine males and fifty-eight females, were involved between 6 and 19 June 1949. When the café from which the ice-cream was obtained was visited, it was found that two employees were unwell, one was off ill with sore throat and another was working though suffering from this condition. A group A type 23 *Streptococcus* was obtained from the waitresses and from many, though not all, of those affected. The ice-cream was examined later on in the course of the outbreak, but no haemolytic streptococci were recovered from it at this stage.

Though group A haemolytic streptococci are the main human pathogens, group B strains may, however, on occasion give rise to infection. Ghoroury(43) has made a comparative study of group B streptococci of human and bovine origin, and has come to the conclusion that the bovine strains are serologically distinct from human. He believed it was justifiable to conclude that group B strains found in man are usually human parasites independent of any bovine sources, and the bovine strains are seldom, if ever, derived from human sources.

STAPHYLOCOCCAL INFECTIONS

Staphylococcal poisoning outbreaks associated with milk and milk products have occurred from time to time. Thus Drysdale(44) has described a large outbreak of staphylococcal food poisoning caused through the agency of goat's milk. Two strains were isolated, one from a sample of unboiled milk and another from a goat with mastitis. Both organisms belonged to the same phage type 6/47. A monkey fed with the boiled infected milk developed symptoms of vomiting and diarrhoea. Again Rosati(45) gave a detailed account of the literature on staphylococcal food poisoning. Furthermore, he examined ninety samples of fresh milk as supplied to the city of Perugia in Italy. From these he obtained fifty-four manitol-fermenting strains of staphylococci from which, after culture in broth, filtrates were obtained and tested for enterotoxin by the intracardial injection of kittens with 2–4 ml. Only one staphylococcal strain gave a filtrate which produced the symptom-complex of vomiting, diarrhoea and collapse in the kitten.

Evans, Buettner & Niven(46) have made a further study of methods for identifying staphylococci associated with food poisoning. They have already indicated that the capacity to produce enterotoxin may be limited to staphylococci which are members of the coagulase-positive group. They agree with Dack(47) that the most satisfactory method available for detecting the ability of a culture to produce enterotoxin appears to be the controlled feeding of large doses of culture

supernatant to rhesus monkeys. In the present series of experiments twenty-four coagulase-negative cultures were fed to a total of fifty-nine monkeys without producing any symptom of illness. In contrast to these results when two coagulase-positive cultures recently isolated from food, involved in food poisoning, were fed to four monkeys all of them became violently ill. Four out of five coagulase-positive strains isolated from foods which were not associated with food poisoning also produced similar effects on monkeys, and a further five out of seven strains from human infections were also toxic to the test animals. The authors conclude that there appears to be increasing evidence that the coagulase-positive staphylococci represent a distinct group of micro-organisms, but the final criterion for the production of enterotoxin must be the feeding of monkeys with the filtrate.

BRUCELLOSIS

This disease in its relation to human infections still commands world-wide attention. Several new text-books or new editions have been published, by Harris(48) in the U.S.A., by Signorelli(49) in Italy, by Otero(50) in Puerto Rico, and a symposium by the American Association for the Advancement of Science(51). In addition, the present knowledge regarding this disease has been summarized by Kaplan(52). This author gives the following statistics for different countries of the world. In 1949 the number of cases officially reported was:

France	1400	Peru	491
Italy	9426	Spain	5484
Malta	910	U.S.A.	4124
Mexico	1369		

Further, the number of undiagnosed and unreported cases must have been very much greater than the official figures, and he states that despite the fact that though only 4000–6000 cases are reported each year in the U.S.A., reliable estimates put the figures at between 40,000 and 100,000. The same must apply to other countries as well, and when one considers the chronic nature of the disease the implications of these figures become more apparent. Kaplan also states that a survey by W.H.O. on the prevalence of brucellosis in animals has yielded only general information. The disease, however, is widespread in cattle, sheep and goats of the Mediterranean countries and Latin America. In the United Kingdom, and in northern and central Europe, except for Scandinavian countries, between 15 and 20% of cattle herds are infected, while for the U.S.A. the figure is approximately 16%. As far as is known, the disease is of little importance in Africa and Asia except where herds of European cattle have been introduced, while in India native cattle have shown a fairly high rate of infection (10–50%). In the *Report on Animal Health Services*(53) issued by the Ministry of Agriculture, mention is made that under the Brucellosis Melitensis Order of 1940, four cases in which an organism indistinguishable from *Br. melitensis* had been investigated between 1940 and 1947. In 1948, *Br. melitensis* was again recovered from the milk from three more herds. Movement restrictions were applied and a veterinary inquiry was carried out. The organism was recovered from the milk of three cows in a herd in Shropshire, and from the milk of one cow in each of the other two herds. The infected animals were thereupon slaughtered.

Stableforth(54) discussed the prevalence, spread of infection, pathogenesis, and control of brucellosis in animals and man. Details are given of the eradication schemes in Norway, Denmark, Finland, the U.S.A. and Great Britain, and of the effect of vaccination with strain S. 19. An epidemiological study of brucellosis in Minnesota has been made by Magoffin, Kebler, Spink & Fleming(55). An analysis of the data on 268 human cases demonstrated that *Br. abortus* was the causative organism in about 85% of the cases. The remaining infections, divided almost equally between *Br. suis* and *Br. melitensis*, occurred chiefly in meat-packing plant employees, though a few cases occurred in farmers. The agglutination test in dilutions of 1/80 or greater was positive in 267 cases with one exception, and in this case a positive blood culture was obtained. Fleming & Roephe(56) have also discussed the relation of human and bovine brucellosis in

Minnesota. During the period 1937–47 the reported rate of human brucellosis has increased 4–10 times in districts not under the area plan of control, while they have not increased in the areas under control. The area control plan includes blood testing of all cattle, slaughter of positive reactors, calf vaccination, restrictions of importations, and retesting of herds at intervals until they have passed three consecutive negative tests. Dalrymple-Champneys(57) reviewed the position in England and Wales in great detail. His analysis was based on the cases reported between the years 1914 and 1949. For the last 10 years there has been an average of forty-six cases annually. He believed that this figure was much too low and that many cases were undiagnosed. In his series of cases there were 592 males and 357 females, and after excluding individuals exposed occupationally there were 490 males and 336 females. Seven patients were under 5 years of age and only forty-six were under 10 years, the decennium with the highest incidence being 30–39 years. The author goes on to discuss the symptomatology and clinical laboratory methods of diagnosis. The mortality in the series was nineteen cases; five, however, had other diseases unconnected with the *Brucella* infection and two were markedly enfeebled by old age. The same author (Dalrymple-Champneys(58)) has contributed a further paper on an epidemiologist's reflexions on some laboratory tests for brucellosis. Smith(59) has described the epidemiology of undulant fever in north-east Scotland over the previous 22 years. A total of 285 cases have been diagnosed with an annual average of 12·9 for 22 years and 16·7 for the last 12 years. This gave an incidence of two cases per 100,000 of the population per annum. The sex and age distribution of the cases in this area were similar to those given for elsewhere, the ratio of males to females being 1·7 to 1, and a high proportion of the cases occurred in the third and fourth decade. The mortality rate for the series was 1·7 per 100 cases.

BOVINE LEPTOSPIROSIS

Little has been added to the literature on the transmission of bovine leptospirosis to man, and the single report which has appeared records the association of *Leptospira pomona*. Thus Wellington, Stevenson & Ferris(60) have discussed the position of endemic leptospirosis in Victoria, Australia. They state that it is chiefly associated in man with occupational groups in the dairying industry and meat trade, and the organism which is transmitted is *L. pomona*. It seems more likely that this form of leptospirosis is transmitted to man through the handling of infected animals rather than through the ingestion of infected milk. On the other hand, where cases of leptospirosis due to *L. bovis* have occurred, the evidence suggests that infection may be contracted by drinking infected milk as well as by handling infected animals.

RICKETTSIAL DISEASES

(1) *Q fever*

In editorial articles in the *British Medical Journal*(61) and the *Lancet*(62,63), the present knowledge of the epidemiology of Q fever was fully reviewed. The disease is now recognized as being endemic in Australia, in parts of the American continent, Europe, Africa and Asia. In European countries outbreaks have been reported in Germany, Austria, France, Spain, Switzerland and England. It is probable that the disease can be contracted in three ways: through occupational contact, by inhalation of infected dust through residence in proximity to infected cattle, and by drinking raw infected milk. Bell, Beck & Huebner(64) in the U.S.A. examined specimens of blood from various workers by the complement-fixation test using *Rickettsia burnetti*, and found that of employees in an aircraft factory 1·2% gave positive complement-fixation tests, while 5·9, 15·6 and 20·6% of those employed respectively in meat-packing plants, hide and pelt factories, and in dairies gave positive reactions. The effect of drinking raw milk was shown by the fact that 10·7% of those that did gave positive reactions, while only 0·9% of those who did not reacted positively. In England and Wales cases of Q fever have been found in Kent, Devon, Somerset, Gloucestershire, Wiltshire, Denbighshire and Oxfordshire, and have been described by Stoker(65), Harman(66), and MacCallum, Marmion & Stoker(67). There would appear to be numerous foci

of infection therefore in England and Wales, but so far no cases have been reported from Scotland and Northern Ireland, though further investigation may reveal their presence. According to Sidky(68) the story of Q fever can be divided into three stages. The first took place in Australia, the second in the U.S.A., and the third in the Mediterranean area. The author goes on to discuss the clinical picture, the mode of transmission, outbreaks of the disease, and milk as a source of infection. Though the disease is normally transmitted through the agency of parasites, both animals and man can contract it by drinking infected milk. An extensive bibliography is given with this paper. Marmion, MacCallum, Roulards & Thiel(69) have carried out a series of experiments on the effect of pasteurization on the survival of *R. burnetti* in milk which was either naturally infected, or in certain instances had been artificially infected. They showed that in the high-temperature short-time pasteurizing plant a temperature of 154–156° F. for 15 sec. was sufficient to render naturally infected milk non-infectious for guinea-pigs. When, however, the milk was artificially infected with large numbers of organisms the temperature had to be raised to 161° F. before it became inactivated. Milk with added *R. burnetti* required an exposure of 30 min. at 143·5–144° F. in the holding process before it became non-infectious for guinea-pigs. In the report of the Joint WHO/FAO expert group on Zoonoses(70), it is stated that in view of the increasing importance of Q fever it has requested that a preliminary study of the prevalence of this disease throughout the world be undertaken. It has also suggested that local and regional surveys should be made, that diagnostic tests should if possible be simplified, that vaccination procedures should be initiated, and that the effect of chemotherapy should be studied.

(2) *Brazilian spotted fever*

Another rickettsial disease, Brazilian typhus fever, due to *R. rickettsia* has been shown to be transmissible by milk. De Magalhaes(71) has been able to demonstrate the presence of the virus in the milk of goats. In many places in the interior of Braxil goats are a substitute for cows as a milk supply for children and adults. These animals are apparently a good reservoir, since the virus can be recovered from the blood and serum from the infected gives a positive Weil-Felix reaction. Experiments have shown that the virus can go through the normal mucosa of the gastro-intestinal tract of dogs causing the disease, and, therefore, it is presumed that it can do likewise after ingestion by human beings.

VIRUS DISEASES

The possible significance of milk and water in the spread of virus diseases has been discussed by Brown(72). Infectious hepatitis, foot and mouth disease, epidemic diarrhoea of the new-born, and poliomyelitis, are listed as being occasionally transmitted through this agency. There has been no recent work confirming the virus theory of the etiology of epidemic diarrhoea of the new-born, and the transmission of foot and mouth disease to humans by milk must be a rare occurrence. Lipari(73) gave a detailed account of an outbreak of twenty-three cases of poliomyelitis amongst the 3000 individuals in the north-east part of Delaware County, New York State, whereas in the rest of Delaware County there were nine cases amongst 42,000 persons. A survey in the area where most cases had occurred showed a possible further seventeen with non-paralytic symptoms. The evidence that the disease was partly milk-borne rested on the fact that, if cases infected by direct contact were excluded, then 86% of the paralytic and 92% of the non-paralytic had consumed milk from the presumed infected source, whereas only 30% of the uninfected controls obtained their supplies from the same source.

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REFERENCES

(1) Feemster (1951). *Amer. J. Publ. Hlth*, **41**, 1275.
(2) Savage (1949). *Brit. J. soc. Med.* **3**, 45.
(3) Kay (1950). *Nature, Lond.*, **165**, 144.
(4) Tullis (1950). *J. R. sanit. Inst.* **70**, 373.
(5) Gibson (1950). *J. R. sanit. Inst.* **70**, 130.
(6) *W.H.O. Tech. Rep. Series*, no. 40, p. 28.
(7) *Report Ministry of Health, Ann. Report of C.M.O. for* 1949 (1951), p. 99.
(8) Wilson (1942). *Pasteurization of Milk*, p. 23. London: Arnold and Co.
(9) *Report to Med. Res. Coun.* J. 479 (1949), **47**, 337.
(10) Chalmers (1950). *Health Bull.* **8**, 10.
(11) Francis (1950). *Lancet*, no. 258, p. 34.
(12) Francis (1950). *Nature, Lond.*, **165**, 102.
(13) Reilly (1950). *J. Hyg., Camb.*, **48**, 464.
(14) Kerr, Lamont & McGirr (1949). *Vet. Rec.* **16**, 466.
(15) Hedvall (1949). *Nordisk Med.* **42**, 1215.
(16) Meara (1950). *S. Afr. med. J.* **24**, 593.
(17) Henning & Aswegen (1950). *J. S. Afr. vet. med. Ass.* **21**, 27.
(18) Paterson & Winmill (1950). *J. comp. Path.* **60**, 219.
(19) Kästli & Binz (1949). *Milchwissenschaft*, **11**, 391.
(20) Kauffmann (1951). *Enterobacteriaceae*, p. 96. Copenhagen: Ejnar Munksgaard.
(21) Felix & Anderson (1951). *J. Hyg., Camb.*, **49**, 349.
(22) *W.H.O. Epidemiological and Vital Statis. Sect. Rep.* (1950), **3**, 296.
(23) Sartorius (1950). *Z. ärtzl. Fortbilding*, **44**, 149.
(24) Werner & Zöckler (1949). *Z. Hyg. InfektKr.* **129**, 218.
(25) Cockburn, Jameson & Fenton (1951). *Bull. of M. of Hlth. and P.H.L.S.* **10**, 43.
(26) Analysis of Food Poisoning 1941–48 (1950). *Bull. of M. of Hlth. and P.H.L.S.* **9**, 148.
(27) Smith & Buxton (1951). *Brit. Med. J.* **1**, 1478.
(28) Ritchie & Clayton (1951). *Mon. Bull. Minist Hlth Lab. Serv.* **10**, 272.
(29) Taylor (1950). *Publ. Health*, **63**, 168.
(30) Editorial (1949). *Lancet*, **2**, 178.
(31) Cromb & Murdock (1949). *Med. Offr*, **82**, 267.
(32) Hemmes & Van Wesemael (1949). *Ned. Tijdschr. Geneesk.* **93**, 3080.
(33) MacQueen (1949). *Hlth Bull.* **7**, 67.
(34) Jones (1928). *J. exp. Med.* **47**, 965.
(35) Jones & Simms (1929). *J. exp. Med.* **50**, 279.
(36) Jones & Simms (1930). *Science*, **72**, 456.
(37) Wilson & Rosenblum (1952). *J. exp. Med.* **95**, 25.
(38) Wilson & Rosenblum (1952) *J. exp. Med.* **95**, 39.
(39) Wilson & Rosenblum (1952). *J. exp. Med.* **95**, 51.
(40) Farnback (1949). *Hlth. Bull., Melbourne*, p. 2773.
(41) Schoechli (1949). *Schweiz. Arch. Tierheilk.* **91**, 780.
(42) Reekie (1950). *Med. Offr*, **83**, 109.
(43) Ghoroury (1950). *Amer. J. publ. Hlth*, **40**, 1273.
(44) Drysdale (1950). *J. trop. Med.* (*Hyg.*), **53**, 12.
(45) Rosati (1949). *Ann. Sanità publ.* **10**, 756.
(46) Evans, Buettner & Niven (1950). *J. Bact.* **60**, 481.
(47) Dack (1949). *Food Poisoning.* Chicago: University Press.
(48) Harris (1949). *Brucellosis.* New York: Hoeber. Inc.
(49) Signorelli (1949). *L'Infezione brucellare nell'nomo.* Napoli.
(50) Otero (1948). *Studies of Brucella infection in Porto Rico.* Porto Rico, San Juan University.
(51) *Brucellosis. A Symposium.* American Association for the Advancement of Science, Washington, D.C.
(52) Kaplan (1950). *Bull. World Hlth Org.* **3**, 309.

(53) *Report on Animal Health Service for* 1948 (1950), p. 12. Ministry of Agriculture: H.M. Stationery Office.
(54) Stableforth (1952). *Proc. R. Soc. Med.* **45**, 79.
(55) Magoffin, Kebler, Spink & Fleming (1949). *Publ. Hlth. Rep.* **64**, 1021.
(56) Fleming & Roepke (1949). *Publ. Hlth Rep.* **64**, 1044.
(57) Dalrymple-Champneys (1950). *Lancet*, **1**, 429.
(57) Dalrymple-Champneys (1951). *Amer. J. publ. Hlth*, **41**, 152.
(59) Smith (1951). *Hlth Bull.* **9**, 57.
(60) Wellington, Stevenson & Ferris (1951). *Med. J. Aust.* 7 July, p. 15.
(61) Editorial (1950). *Brit. Med. J.* **1**, 473.
(62) Editorial (1950). *Lancet*, **1**, 960.
(63) Editorial (1950). *Lancet*, **2**, 630.
(64) Bell, Beck, & Huebner (1950). *J. Amer. med. Ass.* **142**, 868.
(65) Stoker (1949). *Lancet*, **1**, 178.
(66) Harman (1949). *Lancet*, **2**, 1028.
(67) MacCallum, Marmion & Stoker (1950). *Lancet*, **2**, 960.
(68) Sidky (1950). *Bull. World Hlth Org.* **2**, 563.
(69) Marmion, MacCallum, Roulards & Thiel (1951). *Bull. Minist. Hlth Lab. Serv.* **10**, 119.
(70) World Hlth Organ. (1951). *Tech. Rep. Ser.* no. 40, p. 13.
(71) Editorial (1949). *J. Amer. med. Ass.* **140**, 492.
(72) Brown (1949). *Amer. J. publ. Hlth*, **39**, 764.
(73) Lipari (1951). *N.Y. St. J. Med.* **51**, 362.